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THE SCIENTIFIC BASIS OF MULTIPLE SCLEROSIS

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Chapter 1

Clinical Aspects of Multiple Sclerosis

Multiple sclerosis (MS) is a disease affecting the mature central nervous system (CNS) in which more than one lesion is found disseminated in time and space. Frequently there are successive relapses with periods of remission, but in individual patients, the course of the disease can be remarkedly variable. The age at onset of neurological symptoms ranges from 10-50 years, with a maximum incidence at about 25 years of age. MS attacks women more frequently than men, and women are affected slightly earlier. Damage is largely restricted to the white matter of the CNS where lesions consist of fibrous plaques of demyelinated nerve tract, in which axons are frequently spared. The plaques tend to be distributed at sites of preference, for example, frequently following the contours of the third and fourth ventricles. Zonal plaques may be located immediately beneath the pia matter, particularly in the sulci of the cerebral hemispheres (1). Some of the plaques, particularly in the cortex, may be sub-clinical or silent, and only become apparent at post mortem or on EMI scanning (2). Another site of predilection is the optic nerve, but here clinical signs relate more closely to demyelination of the nerve. Plaques of demyelination in the brain stem and spinal cord are also associated with localizing signs, e.g. paraplegia or incontinence.

Epidemiology

MS is not uniformly distributed in the world (3). The incidence is much greater in temperate zone (roughly 40° to 60° north or south of the equator) than in subtropical or tropical zones. There are low risk areas (less than 19 per 100,000) such as South America and Africa, and other areas such as the Northern United States and Europe, where the incidence is high (above 40 per 100,000). There are also centres of even higher concentration, for example, 128 per 100,000 in the Orkney and Shetland Isles. Several studies have focused on the incidence of MS in immigrant populations moving from high to low risk areas (or vice versa). The migrant appears to carry with him the high risk of his country of origin if he emigrates after the age of 15 years (4). Likewise, immigrants into the United Kingdom from Asia, Africa and other low risk areas have a greatly reduced chance of developing MS (5). Dean has pointed out that the epidemiology of MS resembles that of paralytic poliomyelitis. This viral infection is also common in countries with high standards of hygiene, so that the epidemiological findings in MS are consistent with exposure to an environmental factor during late childhood preceding the onset of symptoms. Alternatively, the factor (e.g. virus) may be restricted to the temperate zone and be acquired as a 'slow virus' during childhood.

Clinical

MS is notoriously difficult to diagnose in the early stages of the disease. It has an utterly unpredictable course. A few patients are afflicted with a severe, rapidly progressive form of the disease, and may die from the secondary effects within a few years. However, the majority suffer varying degrees of motor and

sensory disabilities during the course of alternating exacerbations (relapses) and remissions. An individual may suffer a relatively severe attack which subsides within a few weeks or days, then be apparently free from symptoms for months or years until another disabling attack supervenes. Although the aetiology of MS is unknown, the disease can be apparently exacerbated by stress or by intercurrent infections. The degree to which changes in the state of the patient are attributable to functional changes in pre-existing lesions makes clinical assessment of a patient difficult as symptoms may even be intensified by increased body temperature (e.g. a hot bath). Thus, a relapse must be identified as a distinct new sign, and not be due to re-emergence of an older lesion. Rapid changes in signs and symptoms cannot be due entirely to demyelination and remyelination, for the adult CNS is capable of only very limited myelin synthesis. One such reversible factor may be the oedema of the myelin sheath - considered to be an early pathological event - or the temporary action of synaptic blocking factors present in the sera of some MS patients (6). It is estimated that from the time of diagnosis, life expectancy is about 75% of normal, but as already indicated this estimate is not true for all patients. The variability in the course and extent of the disease has led some to suggest that MS is not a single clinical entity, but rather a spectrum extending from a malignant progressive condition to a relatively benign form such as happens in some cases of optic neuritis.

Classification

It will be appreciated that the criteria adopted for the diagnosis, classification and evaluation of the clinical status of the MS patient are of critical importance to medical research into the disease. A scheme for classification of MS cases (7,8) is shown in Table 1.1. According to that strict criteria, progressive cases are kept separately as the pathogenesis may differ from other forms of MS, and it may be difficult to differentiate from familial spastic paraplegia. Although more than 50% of patients with optic neuitis eventually develop MS, for the individual patient there is no sure prognosis. Although the case history may suggest MS, it is not of itself diagnostic (8). Various tests are available to help the neurologist. The most useful has been the visually evoked potential response in which changes in the wave form may be detected in about 70% of MS patients. Abnormal auditory evoked potentials have also been recorded in about the same proportion of patients, but the response pattern is more complex and difficult to interpret than the visual evoked response. Of increasing value is the examination of electrophoretically separated cerebrospinal fluid (CSF) proteins. Oligoclonal bands in the IgG region (see Chapter 7) are found in as many as 90% of MS patients. Other tests involving lymphocyte and macrophage function in response to antigens (e.g. myelin basic protein) and the simplified erythrocyte mobility test in the presence of linoleic acid are controversial and not established procedures (9).

Immunogenetics

The occurrence of significant clusters of MS cases and the slightly increased familial incidence suggest the influence of an inherited factor or possibly common exposure to an infective agent. Thus, for example, Millar (10) has found an increased incidence of MS in Irish agricultural workers. The results of most familial studies from a number of countries suggest that the proportion of affected first degree living relatives of MS patients is about 15-20 times that expected (8). Frequency of the disease occurs in the following order: siblings, parents, children, other relatives. The low rate of concordance in monozygotic twins shows that a simple genetic explanation of MS is not applicable. There is,

TABLE 1.1 Criteria for the Classification of MS Cases

Definite	Remitting and relapsing history with two or more episodes:
	1. There should be evidence of white matter lesions at two or more separate sites in the CNS. The age of onset is between 10-50 years and the clinical history of signs or symptoms should be for one year or longer. There may, for example, be a history of an acute retrobulbar neuritis or paraesthesiae, motor weakness, double vision, unsteadiness or other symptom known to occur in MS, followed by one or more relapses.
	2. Gradual onset of paraplegia may be followed by relapses and signs indicative of disease in the brain-stem, cerebrum or optic nerve.
Probable	1. Original clinical evidence of multiple lesions, suggesting MS, followed by good recovery.
	2. History of one or more attacks of acute retrobulbar neuritis accompanied or followed by pyramidal or other signs, usually mild in degree, with no subsequent relapse.
Possible	1. As for probable but with unusual features and few signs.
	2. Progressive history of paraplegia usually in early middle age.
Optic neuritis	Recurrent retrobulbar neuritis without other features of MS is excluded.

[After McAlpine and co-workers (7); McDonald and Halliday (8)]

however, good - although indirect - evidence of a genetic component associated with susceptibility to MS. It has been found that a single immunogenetic complex determines the strong transplantation antigens of a particular species. This is known as the major histocompatibility complex (MHC). The first human antigens were detected on leucocytes and are therefore known as the human leucocyte antigens (HLA). Detection of HLA initially depended on the availability of agglutinating anti-sera but later a more reliable and convenient lymphocytotoxic test, was developed (11). When lymphocytes from different individuals are cultured together the cells interact and some are transformed into blast cells. This reaction, known as the mixed lymphocyte culture (MLC), was also found to be primarily controlled by MHC genes (12). The MHC antigens are coded for by genes occurring at four sites or loci on the 6th chromosomes (13). The antigens detected by lymphocytotoxic tests on peripheral blood leucocytes are coded for at

the HLA - A, B or C locus and the antigens which are detected in MLC are coded for by the HLA-D locus. Other genes known as IR genes are also found in the same chromosomal region (14). These cannot as yet be directly identified by in vitro techniques but they are related (though probably not identical) to a class of HLA which are serologically detected and are mainly found on peripheral blood B lymphocytes. In the mouse these serologically defined antigens are known as immune response associated (Ia) antigens but in the human they are closely related to HLA-D antigens and are known as HLA-D related (HLA-DR) antigens (15). The exact relationship between IR, Ia, HLA-D and HLA-DR is not clear.

Many human diseases have been found to be associated with HLA antigens of the HLA - A, B, C, D and DR class (14,15). There is probably more than one mechanism involved in these associations but many of them are thought to be due to associations with immune response genes and therefore the closest associations have been found with HLA-D and DR antigens. The associations with HLA-A and B antigens are probably secondary. Two diseases of the nervous system have been studied in detail (Table 1.2). Myasthenia gravis has been shown to be associated with HLA-B8, Dw3 and DRw3 (the w indicates that the assignment of these antigens is still only provisional). The association is strongest in young female patients without thymoma and is not so strong in older patients or in patients with thymomas (15,16,17). This suggests that more than one pathogenic mechanism is involved in the development of myasthenia gravis. MS has shown to be associated with HLA-A3, B7, Dw2 and DRw2. Again the closest association is with HLA-Dw2 and DRw2 or equivalent locally defined antigens (15,18,19). The mechanism by which the presence of these antigens leads to susceptibility to MS is not known. The genetically determined immunological response to an environmental agent is probably in some way responsible. Any simple hypothesis is complicated by the finding that an equally strong but different association is found in Arab patients with MS (20), indicating either that different environmental agents are involved in the development of the disease in different parts of the world or that other, as yet unidentified, MHC genes are responsible for susceptibility to MS.

Summary

Methods for the clinical evaluation of MS patients have improved allowing for the classification of cases. Neurophysiological techniques and the discovery of oligoclonal bands of antibody in the CSF have been of particular value. The classification has been invaluable in studies of inherited immunogenetic factors in patients. MS has been shown to be associated with certain kinds of HLA antigens. As different associations have been found for different races an unidentified MHC gene is probably responsible for susceptibility to MS. This gene locus may be associated with the immune response gene.

TABLE 1.2 Human Leucocyte Antigens A, B, D and DRw Associations with Multiple Sclerosis & Myasthenia Gravis

	PERCENTAGES*										
	A3	B7	B8	Dw2	Dw3	DRw2	BT101	B Group 4	DRw4	BT102	DRw3
Multiple Sclerosis											
N. European Controls	20	17	16	21	23	22	33	33	24	30	17
Patients of N. European Origin	32	37	22	55	18	44	83	83	23	18	17
Arab Controls	24	3	5	-	-	-	37	-	-	88	-
Arab Patients	21	7	3	-	-	-	44	-	-	35	-
Myasthenia Gravis											
Controls	20	17	16	21	23	22	33	33	24	30	17
All Patients	18	23	39	0	33	21	-	-	27	-	30
Young Female Patients without Thymoma	10	14	73	-	-	25	-	-	25	-	65
Other Patients	32	41	27	-	-	33	-	-	41	-	27

(Data taken from references 15-20)

* Figures are in percentages

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Chapter 2

Composition and Metabolism of the Myelin Sheath

The myelin sheath consists of up to 40 lipoprotein lamellae encircling the axon. Thus myelin is particularly concentrated in areas composed mainly of fibre tracts (i.e.) brain white matter and spinal cord, where it accounts for 50-60% of the dry weight. Myelination in the nervous system proceeds in a caudocranial sequence, pathways becoming myelinated in a phylogenetic order. While myelination of all tracts in the human central nervous system (CNS) has taken place by two years of age, histological studies show that fully mature myelin sheaths are not found until the end of the second decade (1). The period of maximum rate of myelination, which follows the period of cellular proliferation, has a different temporal relationship to birth in different species. In man, the maximum rate of myelination in the cortex occurs after birth. Myelin is elaborated by oligodendroglial cells and in the early stages connections can be seen between the tongue of cytoplasm on the outside of the myelin sheath and the processes coming from the cells (2). Each oligodendrocyte can form and maintain between 30-50 myelin segments (3).

Lipids

Myelin is a structure with a relatively low water content of about 40% and purified fractions from human brain contain about 70% of the dry weight as lipid. This unique chemical property facilitates the ready isolation of myelin of high purity by use of conventional density centrifugation techniques (4,5,6). There are only small variations in myelin composition in different species and in different areas of the CNS (7); the membrane has a lipid molar ratio of cholesterol:phospholipid:galactolipid varying between 4:3:2 and 4:4:2 (Table 2.1). Cholesterol is the most abundant lipid in myelin but the sterol is present in all other cellular membranes. Although not exclusive to myelin the most characteristic lipid is the galactolipid, cerebroside. Its concentration is directly proprotional to the amount of myelin in the brain (8). Another characteristic marker of myelin is ethanolamine plasmalogen. The ethanolamine phospholipid in the vinyl form is almost exclusively located in white matter (6). Small amounts of polyphosphosinositides (9) and gangliosides are also present in myelin. The monosialoganglioside G_{M1} accounts for 80-90 mole % of myelin ganglioside (10) in the rat, but an unusual ganglioside, sialosyl-galactosyl-ceramide (G_7) is a major component of human myelin gangliosides (11).

Proteins

The protein composition of CNS myelin is uniquely simple, with two major constituents, basic protein (BP) and proteolipid protein (PLP) making up 60-80% of the total protein (Fig. 2.1). BP accounts for approximately 40% of the total protein of the myelin sheath and has a calculated molecular weight of about 18,200 daltons. Depending on the species, isolated BP contains approximately 170 amino acid residues and has an isoelectric point greater than pH 10.6 (12). In aqueous solution it has an axial ratio of 10:1 and is a prolate ellipsoid (i.e.)

TABLE 2.1 Lipid Composition of Brain Cell Types & Myelin

Lipids (molar ratio)	Human Myelin[a]	Human White Matter[a]	Bovine Myelin[c]	Bovine Axons[c]	Bovine Oligoden-droglia[c]	Rat Astrocytes[b]	Rat Neurones[b]
Total lipid (% dry weight)	78	65	75	13	29	39	24
Cholesterol	133	107	132	67	44	38	28
Total galactolipid	62	57	63	31	20	2	2
Cerebroside	52	46	51	20	12	-	-
Sulphatide	10	9	8	10	2	-	-
Total phospholipid	100	100	100	100	100	100	100
Choline phosphatides	27	29	25	36	48	53	57
Ethanolamine phosphatides[d]	39	33	40	28	23	28	26
Sphingomyelin	14	18	17	18	11	5.5	4.3
Phosphatidyl inositol	1.8	3	1.9	6.6	6.6	5.7	7.1
Phosphatidyl serine	17	15	15	11	7.5	7.3	5.4
Ganglioside µg lipid NANA/ mg dry wt	0.19	1.00	0.50	0.33	0.74	1.80	0.69

a - Data from O'Brien and Sampson (57), who report the water content of white matter to be 75.2% of the fresh wt.
b - Poduslo and Norton (58).
c - De Vries and Norton (59).
d - Total plasmalogen of human white matter and myelin - 36 and 37 mole percent respectively.

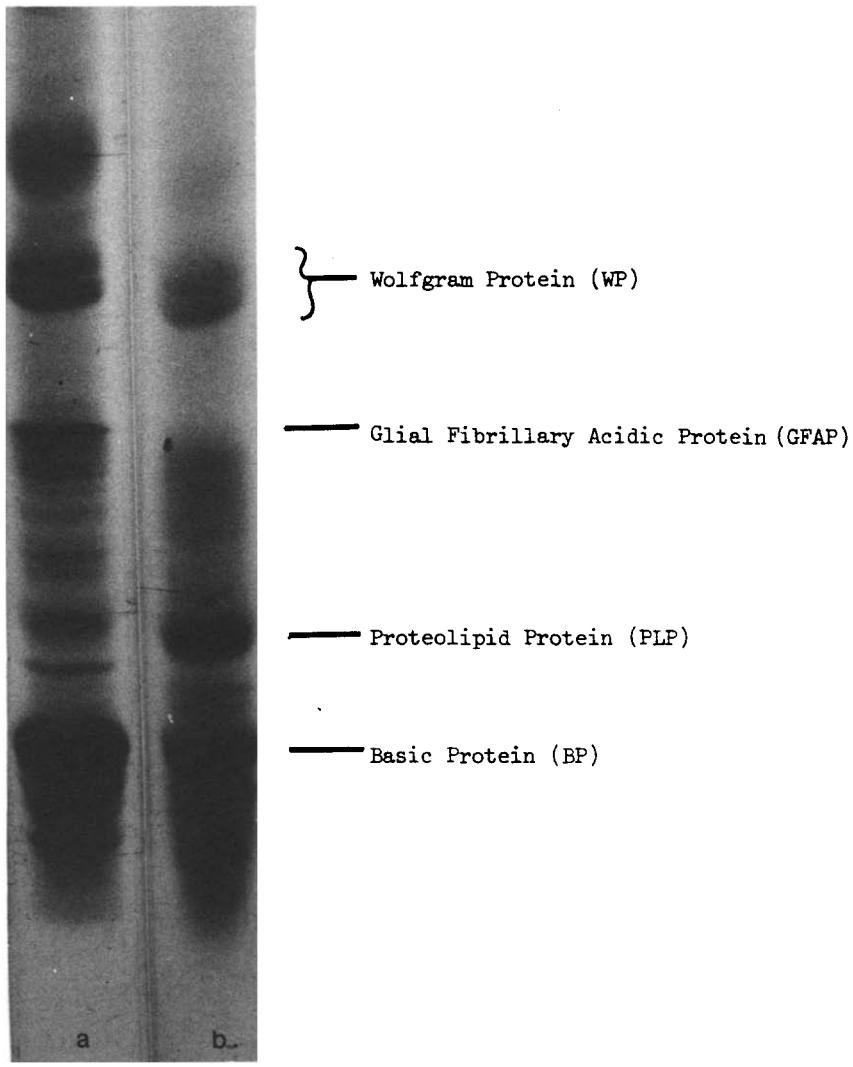
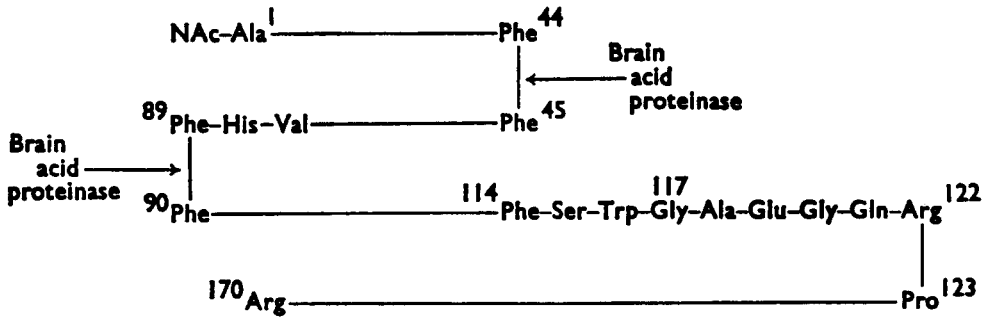


Fig. 2.1 Polyacrylamide gel electrophoresis of brain proteins
(a) Human white matter; (b) Human myelin

a "hairpin" polypeptide, with approximate dimensions of 15 x 150 Å (13). Although circular dichroism and fluorescence measurements show BP in aqueous solution to have very little helical structure (14,15) the in vivo environment of the protein is essentially hydrophobic, thus limiting the interpretation of the data. For comprehensive reviews on the preparation and characterization of BP, see Carnegie and Dunkley (16) and Braun and Brostoff (17).

Biological properties of basic protein. It is well established that myelin BP purified from brain or spinal cord of numerous species induces experimental allergic encephalomyelitis (EAE) in laboratory animals (see Chapter 4). It is a primary demyelinating disease which results from a delayed hypersensitivity reaction to whole BP or encephalitogenic fragments derived from the BP. BP is susceptible to rapid and extensive digestion by trypsin, chymotrypsin, pepsin and pronase (18,19). Hydrolysis by enzymes of the brain and of phagocytic cells is more selective and a mixture of encephalitogenic and non-encephalitogenic peptides are produced. Brain cathepsin D has a special affinity for the peptide bonds of the two Phe-Phe sequences, 43-44 and 89-90, in the molecule (20), and the same three peptides appear to be released by macrophage acid proteinase (Fig. 2.2). BP is also highly susceptible to digestion by the neutral proteinases of polymorphonuclear (PMN) cells (21). Thus the enzymes endogenous to brain tissues may play a rate-limiting role in BP breakdown, while the relapse of fragments by cells of the immune system may be important in regulating the immune response in auto-immune disease. In addition to disease induction, BP gives rise to specific circulating antibodies (22), but complement-dependent antibodies do not have a causal role in the development of EAE (23).



Peptide 114–122 is highly antigenic in guinea-pigs (for further details see Rauch & Einstein, 1974)

Peptide 117–170 has potent encephalitogenic activity in monkeys and is active in producing macrophage inhibitory factor in acute MS lymphocytes

Fig. 2.2 Representation of primary sequence of human myelin basic protein (16).

Proteolipid protein. Attempts to characterise fully the Folch-Lees PLP have been hindered by the presence of anionic phospholipids tightly bound to the molecules. The lipid-free protein is not readily soluble in aqueous media, and requires detergents for solubilization. However, it can be readily succinylated to give a molecule which is soluble in a wide range of aqueous conditions. Analyses of SDS-protein complexes by polyacrylamide gel electrophoresis (PAGE) has established a molecular weight of 30,000 for the major component and 25,000 for the minor component (DM-20) (24). When the protein is prepared by column procedures, the majority of glutamate and aspartate residues are present as amides, which would result in a net basic charge on the molecule. The amino acid composition of PLP

from different species is well documented but work on the sequence is incomplete. Information about the exact molecular shape of PLP is lacking, but in organic solvents the protein has a high helical content (25). Taking the molecular weight of the apoprotein as 23,500 daltons PLP in a spherical form has been calculated to have a diameter of about 38Å . The free sulphydryl groups appear to contribute stability to the secondary and tertiary structure of the PLP and in the native protein these are protected from oxidation by the anionic phospholipids (26). Because of its hydrophobic nature PLP is relatively resistant to proteolysis, but in the presence of the non-ionic detergent, Triton X-100, 80% of the expected digestion by trypsin takes place (27). The oxidized crude proteolipid is more susceptible to tryptic digestion than the unoxidized preparation (28). Both apoprotein and the oxidized proteolipid are digested by thermolysin and α-chymotrypsin and all preparations are attacked by elastase.

High molecular weight proteins. When the total proteins of myelin are separated by SDS-PAGE, a high molecular weight component, resolved as a doublet, is seen in the molecular weight range of 55,000-65,000. This protein fraction is referred to as the "Wolfgram fraction" (WP) (29). The high molecular weight protein fraction comprises a large part of the total myelin protein in the immature brain (30). As the brain matures the pattern changes and the amount of basic and proteolipid protein show a relative increase. A small amount of glycoprotein is consistently found in isolated myelin and accumulates concurrently with myelin formation (31).

Molecular Architecture of Myelin

The application of lactoperoxidase iodination, galactose oxidase labelling and immunochemical techniques, and the use of chemical probes has resulted in a model

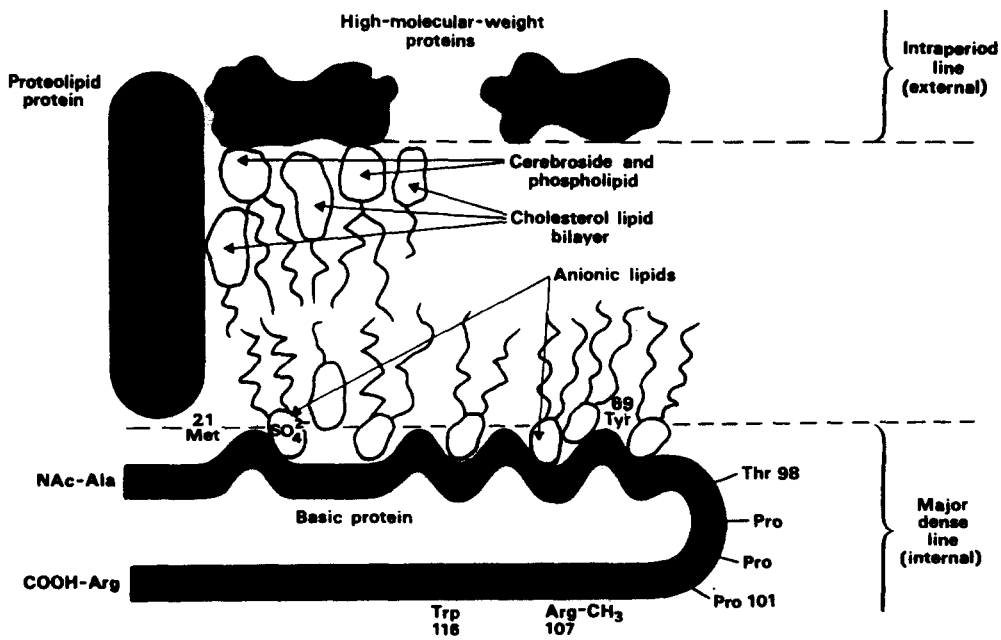


Fig. 2.3 Lipid-protein interaction in unit myelin structure (21).

of the molecular organisation of myelin (25,32). The components of myelin appear to be asymmetrically distributed in the membrane. For the lipid phase of myelin a bilayer arrangement with an intermediate fluid state best fits the data. The action of galactose oxidase points to a localization of cerebroside and sulphatide on the external membrane surface but the localization of phospholipid, cholesterol and ganglioside has yet to be determined. The high molecular weight proteins are considered to be intrinsic components located on the external membrane surface, with part of their polypeptide chains in the lipid phase. PLP is also intrinsic and may be completely embedded in the lipid phase. BP is an extrinsic component localized at the surface of the lipid phase at the cytoplasmic membrane surface (Fig. 2.3).

Enzymes

Compared to other brain structures, myelin is relatively metabolically stable and has low enzyme activity. There are two characteristic enzyme components of myelin. The first, 2',3'-cyclic nucleotide 3'-phosphohydrolase has been used as a myelin marker - 60% of total activity is present in myelin and high levels are also found in oligodendroglial cells (7). A physiological function has yet to be assigned to this enzyme. The second, myelin-specific enzyme, cholesterol ester hydrolase has a pH optimum of 7.2, and may play a role in the control of brain cholesterol ester levels during development. A protein kinase, capable of phosphorylating basic protein, and a neutral proteinase have been located in myelin, but as yet no physiological function has been attributed to either of them.

Development

During myelination the activities of a number of enzymes concerned in the biosynthesis of myelin lipids increase in direct proportion to the rate of myelin deposition. The specific activities of enzymes responsible for the synthesis of cholesterol, phospholipids and galactolipids are found to be highest in the microsomal fraction. The peak activity of cerebroside synthetase in mouse microsomes for either hydroxy or non-hydroxy fatty acids precedes the maximal rate of myelin accumulation by 2-3 days (33,34). Morphological maturation of oligodendroglia in organ culture is accompanied by an increase in sulphatide synthesis (35) and isolated oligodendrocytes are enriched in a number of galactolipid-synthesizing enzymes (36).

The enzymes involved in cholesterol biosynthesis increase in activity during myelination and then decrease (37) and the same pattern has been found for the synthesis of the two phospholipids enriched in myelin, triphosphoinositide and ethanolamine plasmalogen (38,39). Pulse-chase experiments, in which the specific radioactivity of a component in the proposed precursor membrane drops dramatically, indicate that transport of sulphatide between microsomes and myelin may be effected by a soluble lipoprotein (40,41). A different route of transport is proposed for phosphatidyl choline as the specific radioactivity of the lipid in the supernatant is always greater than in the microsomes (42). The phosphatidylinositol and phosphatidyl choline transfer activities in the soluble protein fraction prepared from synaptosomal and myelin fractions were found to be increased seven- and three-fold respectively, compared to whole brain. The synthetic sites and transport mechanisms for myelin proteins have not been determined. After intracranial injection of labelled amino acid, Benjamins and co-workers (43) found that the appearance of radioactivity in all myelin proteins showed a lag relative to total brain proteins. Both in vivo and in slices (44),

the high molecular weight proteins, including the Wolfgram proteins, incorporate radioactivity at a faster rate than basic or proteolipid proteins.

The chemical composition of myelin changes with the age of the animal. As the rat matures, the myelin galactolipids increase about 50% and the phosphatidyl choline decreases by a similar amount (45). In the immature brain the high molecular weight proteins make up the majority of the total. As the brain matures the relative concentration of high molecular weight proteins decreases and the amounts of the basic and proteolipid protein show an increase (31). There is also evidence that accumulation of the BP precedes that of the PLP. It has been proposed that the myelin first laid down by the oligodendroglial cell has a composition which may be close to that of the glial cell membrane (46). The difference in myelin composition from immature brain was found to be a result of the co-separation of a "myelin-like" fraction with the unpurified myelin during the centrifugation procedures. The "myelin-like" fraction accounted for as much as 50% of the crude myelin from 15-day-old rats, but a much smaller amount from adults. The evidence that this fraction is related to myelination is somewhat inconclusive and has been reviewed recently by Norton (7).

Metabolism

Since the myelin sheath contains many tightly-packed lipoprotein membranes with only a very tenuous link to the mature oligodendrocyte, it has always been difficult to consider this structure as a dynamic one. Myelination appears to be very restricted in the adult CNS when deposition of myelin ceases. This concept was supported by the finding of little enzyme activity within the myelin sheath, and in experimental studies turnover of myelin appeared to be exceedingly slow. For example, substantial amounts of labelled cholesterol were incorporated into the CNS of rabbits and chickens only during development. There was evidence that the same [4-^{14}C]-cholesterol molecules remained for more than a year in white matter, while disappearing within a few months from other organs as well as from grey matter. Similar results were obtained with [^{14}C]-serine as a precursor. There was little loss of radioactivity from labelled components measured in whole brain or isolated myelin over a long interval, suggesting that myelin is stable and metabolized as a unit (47). In adult rat brain myelin accounted for only 12% of the total radioactivity 1 day after injection with [^{14}C]-acetate, and after increasing to 15% by 5 days the the absolute amount remained unchanges throughout the experiment. The half life of the myelin fraction was found to be 370 days and in common with long term experiments in myelinating rats, at least 50% of the remaining total brain lipid radioactivity was present in the myelin fraction after 3 months. These results emphasize the higher turnover rates of fractions other than myelin. The fact that some radioactivity is taken up by lipids in adult myelin indicates that a small fraction of adult myelin undergoes rapid metabolism while the rest of the myelin lipid is relatively stable (48).

But evidence against the concept of the myelin sheath as a uniform metabolic entity came from a number of laboratories (49). In particular, Smith (50) injected adult rats with [^{14}C]-glucose, and found significant radioactivity in the myelin fraction, which then underwent slow turnover. Inositol phosphatide and lecithin showed a faster turnover than serine phosphatide and ethanolamine phosphatide, which in turn were metabolized more quickly than sphingomyelin, cholesterol and the galactolipids, cerebroside and sulphatide. Nevertheless, in relative terms, the metabolic activity of myelin is low. The labelling of the polyphosphoinositides with [^{32}P] -phosphate in isolated pure myelin was found to be as great as in whole brain (51) although little synthesis of the remaining myelin phospholipids occurs. In the long term studies of myelin proteins in

young and adult animals both long and short half-lives for individual proteins have been found, but most studies indicate that portions of the basic and proteolipid proteins in myelin are essentially stable, while the high molecular weight proteins have half-lives of 1-2 months (52).

Lipid Exchange in Myelin

The rate of myelin metabolism reflects a balance between net addition and exchange of molecules in the membrane. The results of Sophn and Davison (53) in both young and adult animals *in vivo* showed an uptake of radioactive cholesterol by all subcellular fractions, with an ultimate uniform distribution of the labelled lipid. Studies with cholesterol synthesis inhibitors showed that there was unexpected rapid disappearance of sterol precursors incorporated into the developing myelin sheath on discontinuing drug treatment (54). The loss of incorporated precursors from the myelin was effected by an exchange process and not by conversion to cholesterol within the membrane. *In vitro* studies have also provided evidence of exchange between myelin and other subcellular fractions. Pre-labelled microsomal fractions, incubated with unlabelled whole brain homogenates, transferred 5% of the cholesterol, 4% of the phospholipid and 10% of the cerebroside radioactivity respectively to the myelin fraction (55). In the reverse experiment, 6-10% of the label in each lipid class was recovered in the microsomes. Miller and Dawson (56) have demonstrated transfer of ^{32}P-labelled phospholipids under certain conditions, but myelin was found to inhibit completely these transfer reactions.

Summary

Myelin is a multi-lamellar membrane whose composition is well established. About 70% of the dry weight is lipid - cholesterol, phospholipids and cerebrosides being the dominant components. There are three major protein constituents of which myelin basic protein is the most interesting; some of the myelin constituents are remarkably metabolically stable while other components undergo turnover. Some myelin lipids undergo exchange with molecules from the cytoplasm. There is good evidence that oligodendroglial cells are responsible for the synthesis of myelin. The integrity of the adult myelin sheath thus depends on the continual activity of the sustaining glial cell. There is very active myelination during the postnatal two year period in man, but this decreases slowly and in the young adult it is probable that there is little synthesis of some myelin components. This may explain the age of onset of MS which usually occurs at time when active myelination and the ability to repair denuded axons has ceased.

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Chapter 3

Mechanisms of Demyelination

Although the aetiology of multiple sclerosis (MS) is unknown there is evidence of an inherited genetic factor coupled with a non-specific virus infection and an auto-immune response to a myelin or glial antigen. There is no satisfactory animal model which fulfils all these criteria but there has been extensive experimental investigation of demyelination precipitated by one or more of these causative factors. The most widely studied animal model of demyelination is experimental allergic encephalomyelitis (EAE) which has numerous features in common with MS, and is an auto-immune disease, induced by myelin basic protein (see Chapter 4). The sequence of events following nerve section or the action of neurotoxic agents gives an indication of the process of demyelination in most experimental animal models.

Secondary Demyelination

Wallerian degeneration. If peripheral nerve is sectioned, the part which is isolated from the nerve cell body undergoes degeneration. A consequence of the axonal degeneration is secondary demyelination, and the biochemical findings reflect those of a wide variety of demyelinating conditions, i.e. reduction in the myelin proteins and in the major myelin lipids, cholesterol, cerebroside and sulphatide, and ethanolamine plasmalogen, and an increase in cholesterol ester. Cholesterol ester accumulation, in conjunction with raised lysosomal hydrolase activity, is indicative of active phagocytosis of myelin. When demyelinated tissue, containing cholesterol ester is fractioned by density gradient centrifugation a fraction, lighter in density than myelin, floats on the top of 0.32 M sucrose. This fraction is enriched in cholesterol esters and has a higher lipid to protein ratio than myelin. The time course of Wallerian degeneration is much slower in the central (CNS) than in the peripheral nervous system (PNS), because there is little evidence of cell infiltration from the circulation in the early stages after section. Although the yield of myelin from degenerated rat optic nerves is decreased, the isolated myelin appears to be morphologically and chemically normal (1), apart from a slight increase in cholesterol concentration, and a slight decrease in the amount of ethanolamine glycerophosphatides. These non-specific lipid findings have been seen in other demyelinating conditions. No preferential breakdown of myelin protein constituents occurs. The finding of normal protein and lipid composition in myelin isolated from rat optic nerve 90 days after enucleation, is consistent with morphological evidence that myelin is not broken down until engulfed by macrophages. There is evidence that glial cells also participate in the phagocytosis of both the degenerating axon and the myelin sheath (2). Demyelination in some experimental conditions is independent of inflammatory cells. Chronic administration of triethyltin to rats produces oedema in the CNS and severe paralysis. Myelin recovery is reduced by 25%, but is not accompanied by increased levels of cholesterol ester (3). Absence of phagocytic cells in the CNS is consistent with lack of cholesterol ester formation. A "floating" fraction is observed when myelin is purified from the oedematous

brain of the triethyltin treated animals but apart from a reduction in total protein content, the chemical composition does not differ from that of myelin.

Diphtheria toxin. The direct micro-injection of diphtheria toxin into the spinal cord or optic chiasma produces a focal lesion which shows microscopically severe myelin destruction with axon preservation and some Wallerian degeneration towards the middle of the lesion (4). The toxin itself is neurotoxic, as demyelination occurs before the first appearance of phagocytic cells and heat denatured toxin is innocuous. At a later stage the oedematous lesion resembles histologically the acute MS lesion in that there is destruction of myelin with preservation of axon continuity and a cellular reaction with phagocytosis of myelin debris (5). In contrast to the situation in EAE, there is very little penetration of the sheath or stripping of myelin by cells. Remyelination in the spinal cord or optic chiasma by oligodendrocytes is not apparent after injection of the toxin. Conduction is completely blocked in large lesions and slowed in smaller lesions. There has been little biochemical investigation of the lesion in the CNS, but in the PNS, where the pattern of demyelination is similar, the effects of diphtheria toxin have been investigated *in vitro* (6). No detectable degradation of myelin proteins or sulphatide in sciatic nerves of chick embryos was found after incubation with diphtheria toxin *in vitro* for 4 hours. Instead, the toxin inhibited the *in vitro* incorporation of radioactively labelled leucine into PNS myelin proteins after 1 hour, resulting in a 84% inhibition of synthesis. As the turnover rates of the components of CNS myelin are much too slow to account for the immediate focal demyelination in the spinal and optic tract lesions, other causative factors must apply in the CNS.

Viral Demyelination

A number of experimental diseases exist, with a viral aetiology, which have a demyelinative phase, but which eventually result in an attack on neuronal and axonal elements. Canine distemper which results from injection of a paramyxovirus closely related to measles is the best characterized model of this group. Two types of lesion are seen histologically, demyelinative and destructive, with a higher incidence of the latter (7). The process of demyelination is associated with macrophages, in the presence of virus. Enzyme changes in the demyelinating lesions - increased β-glucuronidase and acid proteinase activity - parallel macrophage accumulation (8). Lymphocytes were excluded as a significant source of the increased enzyme activity as tissues with few lymphocytes had enzyme activities similar to tissues with many lymphocytes. The finding that plasminogenase activity was highest (67% above control) in the tissues with least severe demyelination was considered to be an indication of its action early in the process of demyelination (9). In a study of acute encephalopathies resulting from measles, sindbis or semliki forest virus infection, Bowen and co-workers (10) also found that β-glucuronidase actiivty appeared to be a particularly sensitive index of glial or phagocytic cell reactivity. Another paramyxovirus, designated as 6/94 virus, isolated from cultured brain cells from MS patients, induces an immunologically mediated degeneration of corpus callosum and paraventricular white matter in mice. In adult mouse brain, the lesions induced by 6/94 virus appeared in the following sequence - perivascular cuffing of lymphocytes and histiocytes, followed by parenchymal tissue degeneration that varied from faint staining of myelin to total necrosis of all neural elements (11). In both of these animal models of viral-induced demyelination, axon-sparing is not a feature. Infection of mice with avirulent semliki forest virus also produces a mild form of typical virus encephalitis (12). Histologically the usual features of perivascular infiltration, astrocytic hypertrophy and focal

TABLE 3.1 Experimental Demyelination

Experimental Condition	Morphology of Lesion	Lysosomal Enzyme Activity Increase
EAE	Lymphocytes, macrophages	Cathepsin A, β-glucuronidase
Hyperacute EAE	Lymphocytes, PMN cells	Neutral proteinase, Cathepsin A
Distemper	Astrocytes, lymphocytes	Cathepsin D
Cerebral infarction	Macrophages	Cathepsin D, β-glucuronidase
Wallerian degeneration (Wobbler mutant)	No gliosis No infiltrating cells	Non-selective increase of lysosomal hydrolases
Triethyltin	Oedema	No change
Scrapie	Astrocytes	Glycosidases, acid proteinase

Cathepsin A - carboxypeptidase (pH 5.2)
Cathepsin D - acid proteinase (pH 3.6)

spongiform vacuolation are accompanied by cerebellar white matter demyelination. Increased activity of the lysosomal enzymes acid proteinase, N-acetyl-β-D-glucos- and galactos- aminidase and β-glucuronidase occurred simultaneously with perivascular cuffing, a reduction in brain virus titres, and an increase in serum antibody levels (13). Thus a combination of viral infection and immune mechanisms responsible for viral clearance from the brain may induce biochemical changes which lead to demyelination (Table 3.1).

The search for viral particles in post mortem brain from MS patients has failed to provide evidence to support the hypothesis of viral infection in MS. In addition, transfer of disease with MS brain material has met with minimal success. In this context the experimental spongiform encephalopathy, scrapie, is of interest. A chronic, non-inflammatory and non-demyelinating degenerative disease, scrapie is a prototype of a slow virus infection of the nervous system. Despite extensive electron microscopic investigations, there is no evidence of specific viral structures in the brain (14). The remarkable characteristics of the scrapie

agent - insensitivity to heat, formalin, UV and ionizing radiation - have resulted in a model of an agent devoid of nucleic acid. As it is impossible to separate scrapie from membrane components, it has been suggested that the agent is a portion of aberrant membrane, which upon infection causes the cell to produce more pieces of the membrane containing the same error. There is a lack of a demonstrable immune response to scrapie - no circulating antibody is present and immunosuppression has no effect on the course of the disease (15). But scrapie inoculation does cause a rapid decrease in the percentage of polymorphonuclear neutrophils (PMN) in the peripheral blood of mice. The PMN factor is similar to but separable from the scrapie agent (16). A possible link between MS and scrapie was indicated in a report that the levels of circulating PMN cells in mice were depressed by inoculation of a supernatant fraction (MSAA) from MS brain (17,18). Although there is no cellular infiltration in scrapie-infected brain, significant enzyme changes occur (19). Lysosomal enzyme changes fall into two groups. A number of enzymes have been found to increase 8 weeks after intracerebral inoculation - including β-glucuronidase, acid deoxyribonuclease and N-acetyl- -galactosaminidase. Late in the disease, during the clinical phase, acid proteinase, acid ribonuclease and β-galactosidase increase. The enzyme changes are considered to be secondary as cuprizone treatment, which has a similar histopathological picture as scrapie, produces the same pattern of enzyme changes.

A slow virus infection with neuropathological features more closely related to MS is visna. Visna is a chronic, inflammatory demyelinating disease. The CNS lesions are characterized by inflammation of the meninges and white matter, associated with demyelination and relative sparing of axons (20).

Two animal models, with demyelination as a complicating feature, are of particular interest in that virus enters the nervous system after initial disease in the reticuloendothelial system. Mouse hepatitis virus encephalitis (JHM strain) has some of the features of progressive multifocal leucoencephalopathy (PML), a rare human demyelinating condition, which occurs in individuals with chronic diseases of the reticuloendothelial system or receiving immunosuppressive therapy. In mice non-specific demyelination related to mononuclear cells takes place in the presence of virus, but the loss of myelin is a result of specific infection, not of inflammation. No increase in cholesterol ester was detected, consistent with the absence of phagocytic cells (21). That myelin loss can occur in the absence of inflammatory cells has been demonstrated experimentally in the white matter of animals, administered chronically with triethyltin (3). Marek's disease in chickens results from herpes virus infection of the lymph system. Invasion of the PNS by inflammatory cells which destroy myelin in the same manner as in EAE in the CNS, is a secondary complication of the disease. From the studies of animal models of demyelination and viral infection, it is evident that many have features common to MS but in no case can any direct link be found.

In Vitro Demyelination

On the basis of animal models of demyelination, it appears that proteolytic and lipolytic enzymes, or their products, are the most common mediators of myelin breakdown. For this reason there has been detailed investigation of the action of these agents on intact nervous tissue, isolated myelin and its constituent lipids and proteins. The molecular mechanisms and temporal events in the demyelinating sequence have been studied.

Intact nerve. The action of phospholipase A and lysolecithin on intact nervous tissue has been investigated in the PNS by Hall and Gregson (22) and in the CNS by Blakemore and co-workers (23). Trypsin has also been found to exert activity

on intact sciatic nerve (24). Injection of the lipolytic agents under the perineurium of the sciatic nerve in vivo results in demyelination within 30 min spreading along incisural and paranodal channels. The myelin sheath disappears in the lesion by 96 hours and is replaced by debris-filled cells, resembling macrophages. Phospholipase A_2 produces an identical effect, demonstrating ultrastructurally the progressive disruption and removal of the lamellar sheath. Injection of lysolecithin into the spinal cord produces a similar lesion in the CNS with lamellar splitting and expansion of the myelin structure is also seen when lysolecithin is added to CNS myelin in vitro. It is produced by the addition of approximately half the amount of lysolecithin required to solubilize myelin completely and its appearance can be correlated with loss of basic protein.

When the epinural and perineural sheaths are split to allow penetration of the intact sciatic nerve by trypsin, the major dense line is split and the lamellae of the intraperiod line are separated. After 24 hours digestion by 1% (w/v) trypsin, with appropriate controls, the basic proteins (P_1,P_2) were found to be detectable while the major myelin protein (P_0), a glycoprotein extremely insoluble in aqueous solution, was not completely digested (25).

Purified myelin. The activity of crude Crotalus atrox venom and phospholipase A_2, (purified from the venom) on myelin has been investigated by Coles and co-workers (26). Freeze-dried myelin was suspended in buffer by sonication; the constituent phospholipids were more extensively hydrolyzed by crude venom than by the purified enzyme. The rates of hydrolysis of lecithin and ethanolamine phosphoglyceride were similar, but somewhat lower than the rate of hydrolysis for the acidic serine phospholipid. The products of the reaction were found to be associated with the myelin under these conditions. But the rates of hydrolysis of the phosphoglycerides in myelin were slower than that of the pure phosphoglycerides in aqueous medium and a high concentration of enzyme was required to degrade all the substrates in myelin.

In most published experiments the basic protein (BP) of isolated myelin has been reported to be susceptible to digestion by trypsin. In the main, myelin preparations were water-shocked. The studies of Banik and Davison with trypsin showed loss of lipids and BP from myelin, but there was no alteration in the ultrastructure of the myelin sheath (27). The co-operative action of phospholipase A_2 in the presence of trypsin resulted in extensive loss of both BP and proteolipid protein (PLP), and conversion of myelin phosphoglycerides into the corresponding lysocompounds; splitting of the myelin lamellae was observed in electron micrographs. On the other hand, Schafer and Franklin (28) with a modified fractionation method, designed to minimize membrane damage, found that the BP in intact myelin or in the lipoprotein phase with sulphatide was not hydrolyzed by trypsin or chymotrypsin. Saito and co-workers (29), and London and Vossenberg (30) have demonstrated that the acidic lipids protect the myelin BP (in the region of amino acid residues 20-113) from hydrolysis by trypsin. Furthermore the BP in intact brain in striking contrast to that of isolated myelin is shielded from its antibody (31).

Endogenous Enzyme Activity

In situ degradation of the BP can occur even during very stringent preparative procedures, emphasizing its susceptibility to the action of endogenous enzymes. Three fragments have been found to be generated from BP by the action of purified bovine brain acid proteinase (32). Analysis of the fragments showed that the action of the enzymes was restricted to two Phe-Phe linkages at positions 43-44 and 89-90, of the protein (33). The isolated fragments retained their ability to induce EAE in guinea pigs and rabbits. When incubated with brain extract at

neutral pH minimal digestion of purified BP takes place (34). In acute lesions of EAE, levels of activity of both neutral and acid proteinases are several fold higher than in control brain, but cathepsin A shows the most marked increase and the evidence points to it as a macrophage marker enzyme (35). Purified cathepsin A failed to attack myelin BP but it acts synergistically with cathepsin D, by further degrading one of the polypeptide fragments of BP, Phe^{43}-Phe^{88} (36).

When isolated myelin was incubated in the presence of a soluble extract of brain at pH 3.6 Roytta and co-workers found that there was profuse breakdown of BP and some loss of PLP (37). Incubation at neutral pH did not result in any breakdown. Marks and co-workers (38) showed that incubation for myelin for 7 hours with purified brain cathepsin D resulted in 25% loss of BP and only 5-10% loss of PLP. Under their conditions, incubation of myelin at physiological pH also resulted in endogenous breakdown of approximately 10% of the BP. Purified myelin BP has been found to be an excellent substrate not only for proteolytic enzymes, but also for a protein kinase, which is associated with the myelin fraction. Vaccinia virus also contains a protein kinase, which is located within the viral core. A ten-fold increase in ^{32}P-incorporation observed *in vitro* with added myelin BP, compared with core alone, has been shown to represent phosphorylation of the BP (39). Vaccinia virus can induce a demyelination and it has been suggested that direct viral action on membranes may be one mechanism of action. The enzyme is reactive with a range of BPs, and the relevance of phosphorylation of myelin BP with vaccinia virus protein kinase to demyelination remains to be determined. These results demonstrate that lysosomal enzymes endogenous to brain tissues may play a role in regulating an immune reaction as well as controlling a general metabolic balance.

Immunologically-mediated Demyelination

The phagocytic cells of the immune system, in particular, are supplied with a battery of lysosomal enzyme activities (Table 3.2). When isolated myelin or purified BP is incubated with activated peritoneal macrophage homogenate at acid pH, BP is preferentially broken down and three high molecular weight peptides are produced (Fig. 3.1). There is at least a ten-fold greater digestion at the more physiological pH of 7.6, by human PMN and guinea pig peritoneal macrophage

TABLE 3.2 Lysosomal Enzyme Activity of Mononuclear & PMN Cells

Cell	Cathepsin D* (pH 3.6)	Neutral proteinase* (pH 7.6)	β-Glucuronidase* (pH 5.5)	Phospholipase A_2 (pH 5.5)
PMN leucocyte	0.14	2.09	0.04	+
Macrophage	2.50	0.10	0.20	+++
Lymphocyte	0.16	0.10	0.04	+

Results are expressed as μmoles/mg protein per hour

homogenates. Morphological studies in EAE and MS show myelin disruption before ingestion by phagocytic cells. When isolated myelin is incubated with cell free supernatants from cultures of activated macrophages, BP is preferentially degraded (40). Myelin appears to be vulnerable to attack by at least two neutral proteolytic activities, secreted by the macrophages, a plasminogen-dependent and a plasminogen-independent activity. In addition, the selective digestion of BP at pH 7.6 by intact lymph nodes cells has been reported (41). These reactions constitute a common mechanism of initial myelin destruction in inflammatory lesions, due to the remarkable sensitivity of BP to proteolysis.

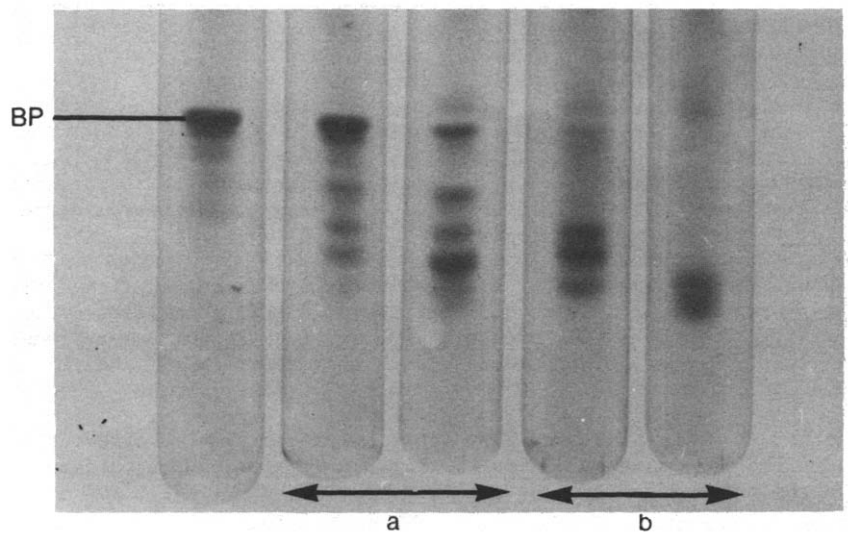

Fig. 3.1 Proteolytic digestion of 40 µg BP by (a) guinea pig macrophages (20 µg protein) at pH 3.6 and (b) human PMN leucocytes (1 µg protein) at pH 7.6. Reaction time 20 and 180 min.

Summary

The sequence of histological and biochemical changes in experimental demyelination are well known. Wallerian degeneration in the CNS is much more protracted than in the PNS. There is loss of myelin components and cholesteryl esters appear. Diptheria toxin and virus induced demyelination have been studied experimentally. Changes in brain hydrolase activity are documented in experimental viral encephalitis in mice and in slow virus infections of animals. In some animal models (e.g. visna) there is demyelination with an inflammatory reaction.

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Chapter 4

Experimental Allergic Encephalomyelitis

Multiple sclerosis (MS) so far as it is yet known occurs only in man. The closest approach to an animal model of the disease is experimental allergic encephalomyelitis (EAE). Autologous, homologous, or heterologous brain or spinal cord, incorporated into an immunological adjuvant, is used to induce EAE. Encephalitogenic activity is due to organ-specific but not species-specific antigenic constituents. Thus brain, spinal cord, the myelin basic protein or its active peptide fragments together with Freund's complete adjuvant (FCA) (killed mycobacteria in paraffin oil) will cause neurological symptoms after intradermal injection. Following transfer of encephalitogenic antigen plus adjuvant into the lymph nodes, there is a short latent period when the mycobacteria stimulate and direct the immune reaction so that specifically sensitized cells are produced. The disease is a delayed hypersensitivity reaction, as the effect can be transferred from sensitized donor animals by means of lymph node cell suspensions (1).

The Clinical Response

Although there is considerable variation in the latent period and sensitivity of different species, neurological symptoms usually appear 10-21 days after injection. The clinical signs, in order of appearance, include ataxic gait, paresis or paralysis of both hind legs, faecal impaction and urinary retention. In monkeys, cats and dogs, varied neurological symptoms develop. Waxing and waning of major signs or occasional clinical remissions are followed by abrupt relapses (2). In rodents the dose and type of encephalitogenic antigen, the nature of the adjuvant, the route of injection, and age of the animal all influence the response. Hyperacute EAE is an extremely severe, uniformly lethal disease produced by active immunization with neural antigen and a double adjuvant system in the highly susceptible Lewis strain of rats (3). In severe cases, animals lose weight before developing ataxia and paralysis of both hind legs. Acute EAE also follows a monophasic course. But a chronic relapsing form has been developed, in which animals show a MS-like picture of remission and exacerbation (4,5,6). The chronic form of EAE is induced in immature inbred guinea pigs by a single snesitization, with a reduced amount of antigen and adjuvant, and is characterized by more than two remissions and relapses before a state of gradual deterioration reduces the animals to a moribund state.

Neuropathology

Acute EAE is primarily an inflammatory condition which is rarely associated with gross lesions in the central nervous system (CNS). The identifying lesion of EAE is in the CNS - predominantly in the spinal cord in small animals but extending to the brain in larger animals, particularly primates (2). Light microscopy shows sharply circumscribed foci of vascular and perivascular inflammation within the brain and spinal cord, affecting white matter more than grey. Gross lesions are uncommon. Perivascular accumulation of fibrinogen, reflecting increased

permeability of the blood brain barrier, has been detected prior to cellular infiltration (7), and exudation of plasma has been noted in the CNS extracellular space. In other experiments, it appears that cerebral vascular permeability only increases after the establishment of histological changes in EAE (8). There are reactive changes involving CNS glial cells surrounding the focal vasculitis, which consist of proliferating microglia (histiocytes) and astrocytes (2). In the acute EAE animal, no gliosis or plaque lesion comparable to that found in MS is seen although astrocytic activity results in deposition of glial fibres. Oedema and swelling of the myelin is the earliest observed change. Sometimes these cells can be seen to phagocytose myelin. However demyelination occurs only in tissue heavily infiltrated with haematogenous mononuclear cells, with the axons generally spared. Perivenous infiltration of mononuclear cells, lymphocytes and small monocytes, starts about 6 to 7 days after sensitization. Plasma cells appear 2 or 3 days later in the perivascular cuff, choroid plexus and meninges (9). In guinea pigs and rabbits, focal meningeal cellular infiltrates are the commonest histological feature (10). The dense accumulation of mononuclear cells in the subarachnoid space with no anatomical connection to white matter raises the question of whether or not the inflammatory response involves antigen in surrounding cerebrospinal fluid (CSF).

The usual lesion consists of predominantly mononuclear cells - histiocytes, lymphocytes and a few plasma cells. Polymorphonuclear (PMN) cells are not uncommon in very early lesions. 90% of the inflammatory cells in the CNS have no immunological commitment to the encephalitogen (11). In the monkey and dog, PMN cells are very numerous and the lesions which may resemble microabcesses (12) are scattered throughout the CNS including occasionally the optic nerve. There is proliferation of astrocytes, but no glial reaction resembling the gliosis of plaque lesion of MS. Demyelination does not occur in the absence of mononuclear cells. In animals with chronic relapsing EAE, old and recent demyelinating plaques are the commonest feature, in the spinal cord, brain and cerebellum (6).

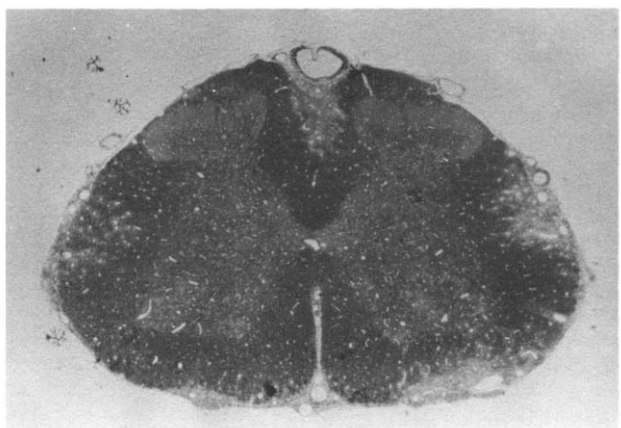

Fig. 4.1 Chronic EAE - 16 weeks - 2 relapses - Note disseminated nature of the lesions - spinal cord at L_7. Chronic plaques are seen in dorsal columns, anterior columns and right lateral column. (Reproduced with the kind permission of Raine and co-workers (44)).

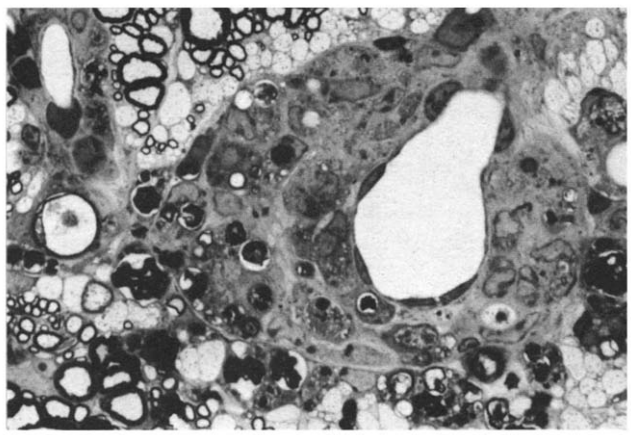

Fig. 4.2 Chronic EAE - 22 weeks post inoculation - 2 relapses. A recent perivascular cuff with a rim of demyelinating axons and macrophages with myelin debris. (Reproduced with the kind permission of Raine and co-workers (44)).

In some the plaques may be large enough to be seen by the naked eye and resemble more closely the pathology of the MS lesion (Figs. 4.1 and 4.2). One of the striking differences between MS and classical acute EAE is the greater number of plasma cells seen in the lesions of the former. In the chronic relapsing model of EAE, a similar predominance of plasma cells is seen in conjunction with relatively greater demyelination. This suggests antibodies are involved in both the animal model and MS.

Immunology

The strongest evidence that EAE is an immunological disease is that it can be transferred with sensitized lymphoid cells derived from the thymus, and that these cells interact with the encephalitogenic antigen residing in myelin (1). A correlation has been found between disease onset and progression, and delayed hypersensitivity reactions such as production of macrophage inhibitory factor(MIF) Peritoneal exudate cells harvested from guinea pigs 9 days after sensitization to spinal cord plus adjuvant are specifically inhibited by extracts of adult rat brain(2).Foetal and neonatal rat brain lack the encephalitogenic antigen and extracts of these do not affect the sensitivity of the cells. Nevertheless there are multiple antigenic determinants in the myelin basic protein (BP) as shown by the studies of Spitler and co-workers (13), who have shown that MIF production and EAE activity can be clearly dissociated. In a study of immunospecifically purified anti-BP IgG, the effective antigenic valence of BP was found to be approximately 4 (14). Another important question is whether oligodendrocytes contain antigens (other than BP) which are capable of producing EAE. Raine and co-workers (15) found that when exogenous myelin BP was removed from bovine oligodendrocytes, these cells with adjuvant did not produce the disease, which suggests that they do not contain a specific encephalitogenic antigen.

Humoral immunity. It is genrally accepted that the induction of EAE is a function of the cellular immune system, and independent of humoral antibody production (2). However in a monoantigenic system (BP), Lebar and Voisin (16) found that the levels of anti-BP antibodies (IgG_1) measured by passive cutaneous anaphylaxis (PCA) correlated with protection from the clinical symptoms, while levels of complement fixing (CF) antibodies (IgG_2) correlated with the severity of the lesions. Some of the CF IgG antibodies induced by BP or whole brain homogenate become attached to the CNS (17). Bornstein and Appel have demonstrated in rabbit EAE sera a CF antibody, which is able to demyelinate cultured brain cells and inhibit primary myelin formation (18). These factors, however, are induced by whole brain and not by the encephalitogenic BP of myelin. It has been claimed that the anti-myelin factors are anti-cerebroside antibodies in the rabbit (19), but Lebar and co-workers have recently reported experiments which show that the demyelinating factor is an autoantibody of the IgG_2 class, which is directed against a myelin antigen that is not BP or cerebroside (20).

Cellular changes. Although in EAE there is no good correlation between morphological changes in the CNS and clinical signs there are marked fluctuations in circulating lymphocyte populations which correlate well with the severity of clinical signs. Traugott and co-workers (21) found that in contrast to circulating B cell and total T cell values, which show little change, early (active or high affinity rosetting) T cell levels show significant decreases which coincide with clinical worsenings in acute EAE. In the EAE animals clinical signs developed on the 11th to 15th day post inoculation. The percentage of circulating active T cells fell at this time from 32.6 ± 12.2% to about 4% prior to death. Throughout the period late T cells showed only minor fluctuations and B cell numbers were slightly elevated. EAE can be suppressed in strain 13 guinea pigs by injection, 7 days after sensitization, of bovine or guinea pig myelin BP. In suppressed animals, between day 17 and 23 post inoculation, transient clinical signs were apparent. Most animals recovered completely, but three out of the twelve had a mild relapse. During the period of clinical response (days 17-23), the percentage of early T cells decreased significantly to 23% of controls. With clinical recovery there was a return in early T cell population to slightly elevated numbers. In animals with later relapses there was a marked decrease in the percentage of early T cells from about 45% to 16%. It was suggested that there might be selective destruction of early T cells or that the loss of active cells may be due to migration into the CNS (which is known to be invaded by mononuclear inflammatory cells). Traugott and co-workers (22) next measured circulating active T cells and assessed the levels in the CNS using ultrasonication to release infiltrating cells from the meninges and surface of the spinal cord. Lymphocytes were not detected in the meninges of normal animals but in sick animals (days 13-24) there was a migration of lymphocytes into the meninges (Table 4.1). A proportion of these were active cells and the percentage (42.4%) was higher than that found in the blood of normal guinea pigs (32.6%). There was no correlation between the percentages of early T cells in the meningeal cell suspensions and the severity of clinical signs. It was therefore concluded that the decrease of early T cells in the circulation was caused by their migration to the target organ, the CNS.

The myelinated fibres of the rabbit eye provide a useful model for the study of immunologically mediated demyelination, and Wisniewski and co-workers have shown that both cellular and humoral activities may be involved (23,24). Supernatant factors (lymphokines), prepared from rabbit lymphocytes activated non-specifically with mitogen, were injected into the vitreous of the eye. In normal rabbits sensitized with FCA there was infiltration of mononuclear cells but no damage to myelin. However, in rabbits sensitized to homologous spinal cord, a demyelinating lesion within the retinal fibres was induced by the lymphocyte products (24).

TABLE 4.1 Lymphocytes in Blood and CNS Infiltrates in Guinea Pigs with Allergic Encephalomyelitis

	(1) Normal Blood	(2) EAE Blood	(3) EAE CNS
Active T cells %	32.6 ± 12.2	14.5 ± 10.6	42.4 ± 13.7
Total T cells %	58.6 ± 12.8	44.5 ± 25.9	43.6 ± 14.9
B cells %	26.5 ± 8.2	29.2 ± 12.9	34.8 ± 11.1*

Results are mean percentages ± S.D.(%).

* Inflated by high levels of monocytes (34%) in the CNS.

Differences are highly significant ($P < 0.001$) between columns 1 and 2 except for B cells where the difference is $P < 0.05$; between columns 2 and 3 ($P < 0.001$) except for total T cells. Guinea pigs were killed between days 13 and 24 after injection with autologous spinal cord with complete Freund's adjuvant.

(After Traugott and co-workers (21,22)).

Primary demyelination could also be induced in the retinal fibres of the normal rabbit eye by anti-CNS antibodies when combined with the lymphokines of non-specifically activated lymphocytes (25). Sera from normal or FCA-sensitized rabbits, were unable to induce primary demyelination even when injected with lymphocyte products. The antibody-dependent demyelination is therefore precipitated by sensitized lymphocytes activated in the CNS by antigen, resulting in the release of factors which attract mononuclear cells and increase vascular permeability.

Changes in the Cerebrospinal Fluid

Examination of the CSF during early or advanced phases of EAE shows a pleocytosis, usually with an initial PMN response followed by a predominance of lymphocytes (2) The concentration of IgG increases in the CSF of rabbits, from 21 ± 3 mg/l to 143 ± 58 mg/l (25). The concentrations of albumin in CSF and plasma respectively were not different in the two groups, and there was no increase in the uptake of ^{51}Cr-EDTA in the brain of animals with EAE (26). This evidence suggests that at least some of the γ-globulin is synthesized within the CNS. Tourtellotte and Parker (27) have shown a positive correlation between the concentration of IgG in MS plaques and apparently normal white matter and the concentration in the CSF and concluded that the increase in the CSF was a reflection of an excess of IgG in the CNS. But a recent report suggests that in EAE in sheep, passive transfer of proteins from serum to CSF is the most likely cause of the protein and IgG

increase (28). As EAE developed, the albumin to globulin ratio in serum and CSF became nearly identical, while in control animals the ratio was 4:1 and 2:1 in CSF and serum respectively. Unlike MS some of the antibody was directed against myelin BP, which appeared in the CSF (6-18 ng/ml) at the onset of symptoms bound as an antigen-antibody complex. Antibody to BP also appeared in the CSF of animals without clinical or histological EAE, but no free or bound myelin BP was detected.

Biochemistry of Demyelination

In EAE demyelination only takes place in the presence of inflammatory cells, and macrophages are seen to penetrate and engulf the myelin sheath (29). Although active demyelination is associated with cellular exudates in 89% of acute cases of MS, evidence of active stripping of myelin sheath by phagocytic cells is absent. In EAE-affected brain, the infiltrating mononuclear cell surrounds the myelin sheath, which becomes hydrated and splits at the intraperiod line. The mononuclear cells invade the sheath at the mesaxon, stripping off the outer vesicularized lamellae, and finally the myelin fragments are phagocytosed and transformed into globoid lipid inclusions. An important observation was made by Yonezawa and co-workers (30) who demonstrated with time lapse cinematography that endogenous phagocytes were responsible for the attack on myelin when sera with myelinotoxic activity from animals with EAE or experimental allergic neuritis (EAN) was added to a tissue cutlure.

An indication that proteins are the first site of attack in the demyelinating process came from the histochemical finding that proteolytic activity was increased in periventricular white matter in guinea pigs before and during the development of lesions (31). Support for this hypothesis is based on the observation of increased incorporation of radioactive amino acids into brain protein in both myelin and non-myelin fractions in slices from guinea pigs with acute EAE (32). The increase in protein synthesis could be attributed to active synthesis in inflammatory cell. On the other hand, in studies of amino acid activation by tRNA synthesis in rabbit, there was a marked increase in activation of some amino acids in the early stages of EAE prior to cell infiltration. The most likely candiate for the initiation of the demyelinating process is BP. Selective loss of this protein in MS plaques is associated with raised proteolytic activity (33) and it must be protected from the action of acid proteinase while it is being purified from brain tissue.

There is well documented information on the raised proteolytic enzyme activity in the CNS in EAE, which has been reviewed recently by M.E. Smith (34). In rodents, where inflammatory lesions in the CNS are not accompanied by extensive demyelination, activity of acid proteinase is increased 28%, neutral proteinase only 12% and cathepsin A 258% in comparison to controls. In the rat the lesions are microscopic and diffuse, consisting predominantly of lymphocytes and macrophages. Cathespsin A, the carboxypeptidase which acts synergistically with cathepsin D (acid proteinase) is considered to be a marker for macrophages as the specific activity of this enzyme in lymph nodes is 30 times that of brain stem. In the hyperacute microscopic lesions of the monkey, where inflammatory lesions contain PMN cells as well as macrophages, acid proteinase activity is increased by 159%, neutral proteinase (enriched in PMN cells) by 239% and cathepsin A by 400% of control CNS values. Myelin BP is highly susceptible to digestion by the neutral proteinases (elastase and cathepsin G) of PMN cells (35). None of these enzyme changes is seen before 7 days after injection with spinal cord, that is before the onset of clinical symptoms. Buletza and Smith found increased proteolytic

activity throughout the pH range 3.5-8 in spinal cords from rats with acute EAE and the optimum pH for acid hydrolysis of myelin BP coincided with that of haemoglobin (36).

BP is the myelin protein which is most susceptible to proteolysis but lipid components of the myelin are also subject to enzyme hydrolysis. Much of the membrane is comprised of lipids and a number of investigations have revealed changes in lipolytic enzymes in EAE. Woelk and co-workers (37,38) found enhanced activities of phospholipases A_1 and A_2 (which are localized in the microsomal and mitochondrial fraction, respectively) in rat brain during the acute stage of EAE. The activity of phospholipase A_1 was increased by approximately 35%, with the same order of magnitude for a wide range of diacylglycerophospholipids. Phospholipase A_2 has a higher affinity for the diacyl than the plasmalogen analogue and the activity was 25% higher in EAE brain. The activity of both enzymes returned to control level during the recovery stage. Another important myelin lipid is sulphatide, which can be hydrolysed by sulphatases. Vasan and Bachawat (39) have reported that arylsulphatases A and B in rat CNS show a maximal activity (3 x control) after the onset of EAE, with a rapid return to normal during the acute and recovery stage. These results agree with their earlier finding of increased incorporation of sulphate into sulphatide during the acute stage of EAE suggesting a higher turnover rate. The increase in arylsulphatase A activity during the acute stage of EAE, observed by Maggio and co-workers (40) was less significant with a slower rate of return to control values.

Although significant amounts of proteolytic enzymes are present in normal CNS tissue, brain lysosomes are very stable and thus the intrinsic enzymes are not readily available. To date one cannot conclude that acid and/or neutral proteinases initiate the process of demyelination as BP-deficient myelin has not been isolated from EAE or MS brains. However the increases in proteolytic activity are several fold greater than those of lipid degradative enzymes. The increments in enzyme activity in EAE brain are considered to be the result of entry of phagocytic cells into the CNS as a wide spectrum of lysosomal enzymes are affected, including β-glucuronidase and acid phosphatase, and because the timing and extent of the enzyme increases correlate with the inflammatory response

Summary

EAE is a delayed hypersensitive condition in which active T-lymphocytes are an important part of the pathological reaction (for reviews, see 41,42). In the acute condition in rodents perivascular cuffing is a predominant feature but unlike MS there is relatively restricted demyelination. Circulating antibody to the antigen-myelin basic protein affords protection rather than initiating a neurotoxic effect. With the onset of paralysis the level of active T cells decreases concomitantly with the appearance of such cells in the meninges of the affected guinea pigs. In rats developing EAE, there is the appearance of myelin fragments in the blood. Indirect evidence suggests that myelin BP or fragments in normal rats and animals with EAE may act as immunoregulatory factors, influencing development or susceptibility to this auto-immune disease. Whether endogenous myelin BP exerts such a putative immunosuppressive role by activating T suppressor cells is not clear. Although the clinical and pathological changes in acute EAE are not close to those seen in MS, the inflammatory reaction is of importance in both diseases. However, where EAE animals survive, a chronic remitting condition results which has a nearer resemblance to MS and this condition too can be suppressed by myelin BP (43).

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Chapter 5

Neuropathology of Multiple Sclerosis

The extent and distribution of demyelinating lesions in the central nervous system (CNS) varies greatly from one patient with multiple sclerosis (MS) to another. In most cases discrete focal lesions are commonly found, but in a small number there is extensive confluent demeylination (Fig. 5.1). Plaques are

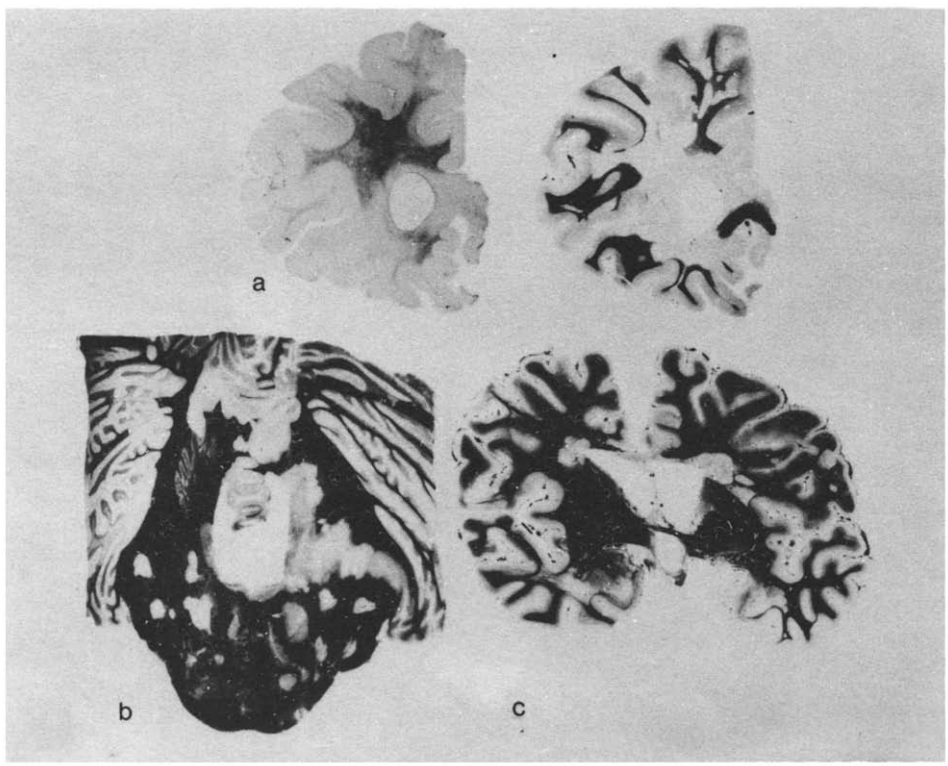

Fig. 5.1 (a) Right frontal lobe. Confluent demyelination, isolated plaques and diffuse gliosis. Heidenhain's myelin - Holzer's crystal violet. x 1.2.
(b) Brain stem. Discrete plaques of demyelination. Luxol fast blue-cresyl violet. x 2.
(c) Numerous plaques present at the angles of the lateral ventricles. Heidenhain's myelin. x 1.0.

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frequently found close to the ventricles, beneath the pia matter in the sulci of the cerebral hemispheres and in the optic nerves. Disabling lesions are found principally in the corticospinal and cerebellar tracts. Since the areas of predilection are in close contact with the CSF it has been suggested that some demyelinating factors may be associated with the CSF (1). In addition it has been observed that lesions are generally centred around a small vein (2); this too implies that an active factor, in this case from the blood stream may be penetrating the brain. Plaques range in size from 1 mm to several cm and it has been suggested that the ratio of microscopic to gross lesions is a good index of acuteness (3). The process of demyelination in the CNS finally results in formation of disseminated plaques of densely packed astroglial fibres enveloping naked axons (Fig. 5.2).

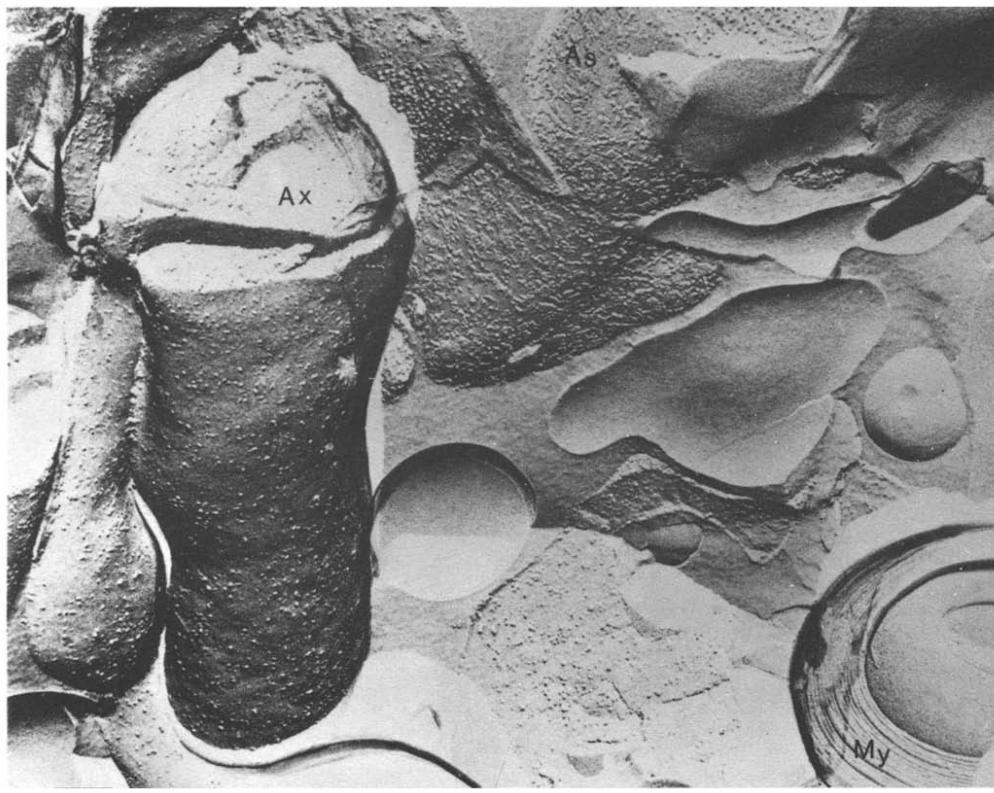

Fig. 5.2 Freeze fracture replica of MS plaque. A naked axon (Ax) is surrounded by processes of astrocytes (As) which are covered with numerous assemblies (arrow). x 30,000. Reproduced with kind permission of M. Dubois-Dalcq.

Active Lesions

Hypercellularity characterizes the active plaque (Fig. 5.3). The significance of perivascular infiltration has been investigated recently by Adams (1)

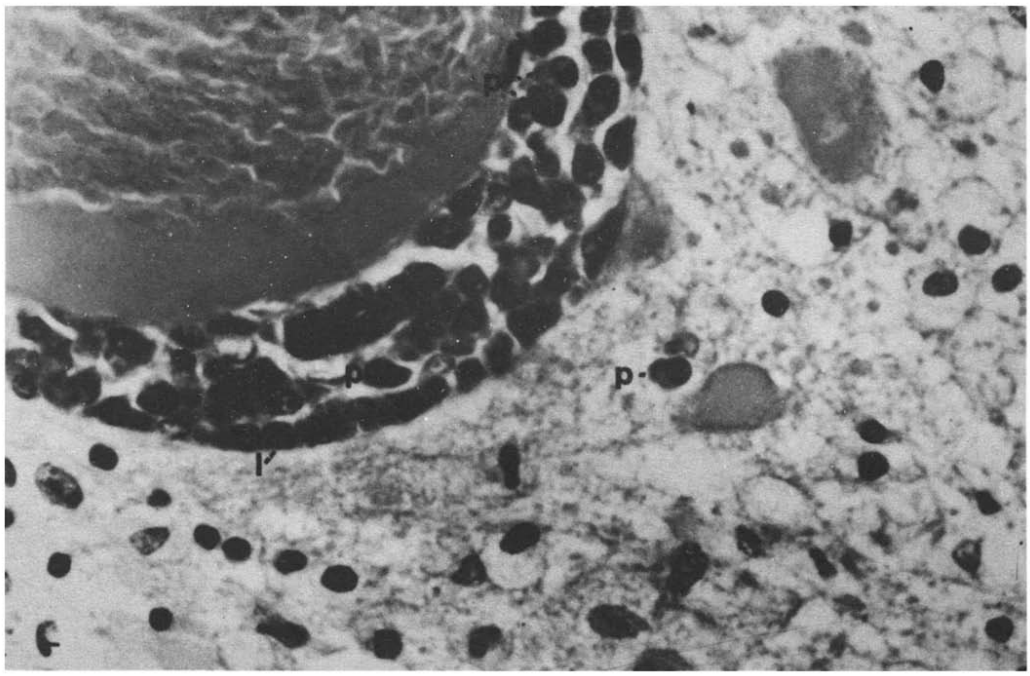

Fig. 5.3 Perivascular infiltration in a demyelinating lesion. Plasma cells (P) and lymphocytes (L) about a blood vessel. Luxol fast blue-cresyl violet. x 850.
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and Jellinger (4) (Table 5.1). In 73% of demyelinating plaques, perivenous inflammatory cells were associated with active demyelination. The duration of illness correlated with decreasing severity of active demyelination and of perivascular infiltration (5). Ultrastructural studies of the cells revealed two distinct types of perivascular cuffs. The acute type, seen at the margin of plaques, consists of lymphocytes and lymphoid cells. The lymphoid cells resemble the blast type of lymphocyte, containing considerable numbers of cell organelles. The chronic type of perivascular cuff consisting mainly of plasma cells (characterised by increased endoplasmic reticulum) and macrophages was seen in the vicinity of the plaque areas. Typical chronic plaque tissue has been found to contain 1772 plasma cells per cubic millimeter of fresh tissue (6). Macrophages participate in the phagocytosis of myelin but no peeling or stripping of myelin sheaths by mononuclear cells has been observed.

There is uncertainty about the identity of some of the cells at the edge of the plaque. On histochemical grounds Ibrahim and Adams (7) considered them to be oligodendrocytes but they may be microglial phagocytes or infiltrating monocytes. Another possibility is that there are phagocytising astrocytes (8). Intact myelin fragments are rarely observed within such cells during demyelination (9). But cytoplasmic inclusions consisting of membrane-bound stacks of curved linear profiles, presumed to be a product of myelin degradation, have been observed in microglia in MS plaques (6). Another feature of the early lesion is the tissue oedema immediately outside the lymphocytic cuff, which appears to affect myelin lamellae and cells and emphasises the inflammatory nature of the process.

Furthermore immunoglobulin-containing cells are significantly more numerous in plaques than in non-plaques and in recent plaques as compared with old plaques (10)

TABLE 5.1 Association Between Demyelination and Pervascular Infiltration in 151 Cases of MS

	No.	No. Associated with Active Demyelination
Perivascular cuffs inside plaques	90	70
Lymphocytic cuffs outside plaques	36	29
Lymphocytic meningitis	62	45
Active demyelination without perivascular cells	20	
Perivascular cells without demyelination	25	

After Jellinger (5)

Lymphocytic meningitis frequently accompanies active lesions (1,5). In his series of 151 cases of MS (19 acute and 132 chronic) Jellinger found inflammatory lesions in the meninges in 62 cases. In 80% of these active demyelination was a feature. The meningitis was characterised by lymphocytic and monocytic infiltrates, often enmeshed in a fibrin network. The number of lymphocytes in the sequestered meningeal areas within sulci often exceeded the number within the brain parenchyma and far exceeds the number of free cells in the CSF (1)(Table 5.2). Indeed, the pleocytosis in the CSF of 60-70% of MS patients with an exacerbation may be an

TABLE 5.2 Lymphocyte Counts in Brain and Cerebral Meninges of 10 Selected Cases of MS

	Cells/cm^2	Cells/Whole Brain or Meninges
Brain	102	1.02×10^8
Cerebral meninges	119	4.76×10^8

Maximum number of free lymphocytes/100 ml CSF = 5.6×10^6

After Adams (1)

indicator of ongoing lymphocytic meningitis. In 63% of active cases of MS (11), perivascular cuffs of lymphocytes have been seen outside plaques in areas of relatively intact or sometimes oedematous myelin. At this stage, the perivascular cuff contains few phagocytic cells and is predominantly composed of small lymphocytes again indicating an inflammatory response. This suggests that the demyelination is initiated by an inflammatory reaction but there are alternative explanations.

Ultrastructural Changes

It should be stated that other groups of workers place less emphasis on the involvement of inflammatory cells in the initiation and progression of the demyelinating lesion. In 13% of Jellinger's cases, active demyelination in the absence of inflammatory cells was a feature. Brain biopsy specimens were studied electron microscopically by Suzuki and co-workers (12), Gonatas (13), and Arstila and co-workers (14). The most common myelin abnormality seen was a focal granular degeneration of the sheath, in the absence of adventitious cells. Oligodendroglia showed non-specific degenerative changes, and proliferation of fibrous astrocytes was observed. In the hypercellular zone around an active plaque, inclusion-bearing cells with small, dark nuclei were found. These cells were distinguishable from normal oligodendrocytes and astrocytes by the presence of cytoplasmic processes and the membrane-bound inclusions appeared to be within the smooth endoplasmic reticulum. In an electron cytochemical study, Arstila and co-workers detected increased enzyme activity and the presence of 'myelin-like' material in astrocytic lysosomes (14). In other studies, these reactive cells were considered to be unidentified macrophages. Of particular interest in this context is the observation of Dubois-Dalq and co-workers (15) that in burnt out plaques immunoperoxidase-labelled anti-human IgG stained astrocytic gliofibrils and lysosomes (Fig. 5.4).

Virological Observations

Elevated titres of antibody to measles virus are present in the sera and CSF of a significant number of MS patients (16). This immunological data has raised the possibility of a viral aetiology for the disease. Ter Meulen and co-workers (17), using cell fusion techniques, recovered a parainfluenza type agent from brain cells grown from two MS biopsy specimens. This has not been confirmed but Prineas (9) has observed "paramyxovirus-like" intranuclear filaments in acute lesions from one MS brain. The cells could not be identified but the cytoplasmic fragments contained aggregates of tubules, with an outer diameter of 18-20 nm, a core diameter of 9-10 nm, and cross striations with a repeat distance of 6-7 nm. Similar filaments, present in the mononuclear cells of MS brain lesions have been described by Tanaka and co-workers (18). Corona virus-like structures have also been revealed by electron microscopy, as doughnut-shaped particles, 55-65 nm in diameter in the cisterns of the rough endoplasmic reticulum of cells from one active lesion. Electron microscopy of 3 cell cultures, established from this brain, failed to reveal similar particles. In addition, unusual intranuclear filaments identical with the "paramyxovirus-like" filaments have been found in 0.1-0.5% of the circulating lymphocytes in 5 out of 5 MS patients and 3 out of 8 patients with optic neuritis (19). No similar structures were present in the lymphocytes of 3 neurological and 4 normal controls. Scepticism has been expressed about the viral nature of these structures as Raine and co-workers (20) failed to demonstrate specific paramyxovirus antigens on the filaments by immuno-electron microscopy and in a comparative study of autopsy tissue from a number of unrelated diseases, it has been established that the "paramyxovirus-like" material is not specific for MS and may be related to cellular breakdown (21).

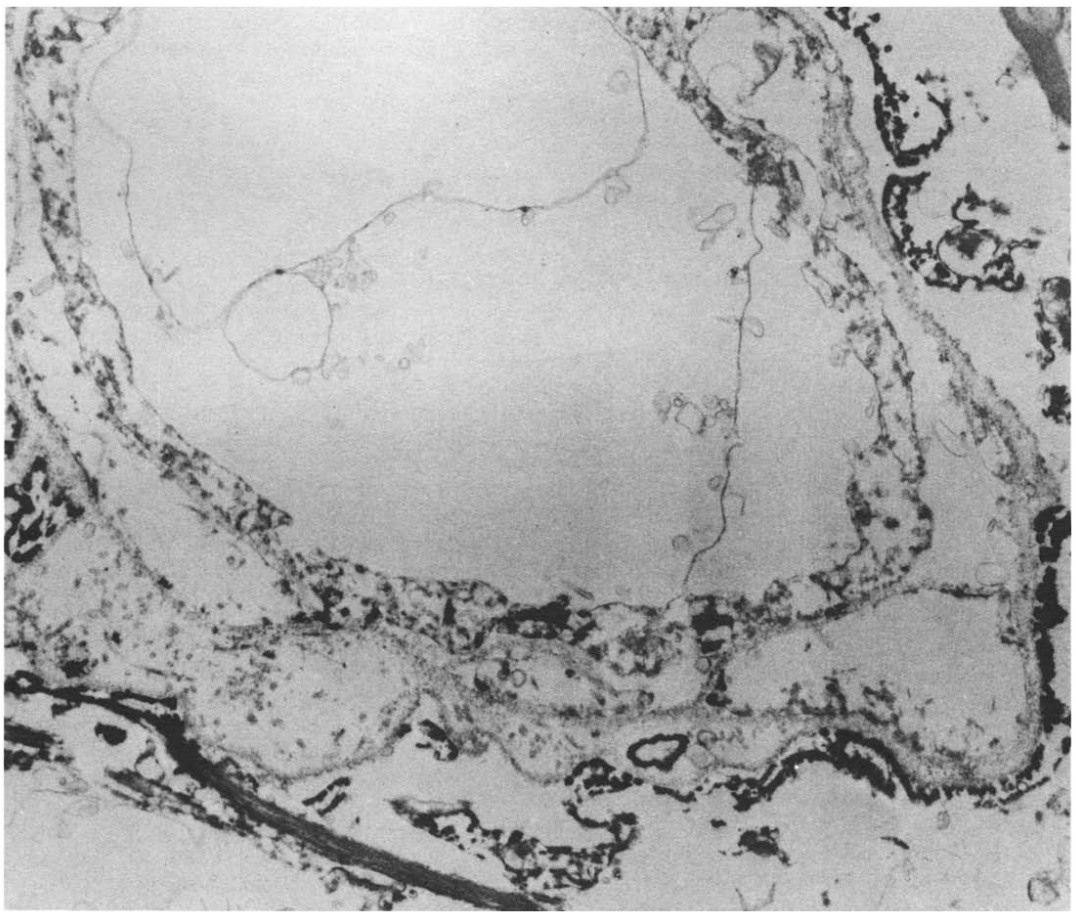

Fig. 5.4 Immunoperoxidase-labelled anti-human IgG in chronic MS. Ultrastructural localization of human IgG in a chronic MS plaque, using the direct immunoperoxidase labelling method. No counter stain with heavy salts. IgG is present on astrocytic membrane closely apposed to the basement membrane around a blood vessel. x 9000. Reproduced with kind permission of M. Dubois-Dalcq.

Definite proof of a viral aetiology for MS must come from successful transmission of the infectious agent to an animal host. However, despite some interesting data indicating a possible infective agent this remains controversial. In these studies injection of specimens from MS patients into inbred mice was observed to induce changes in the differential counts of circulating leukocytes — a decrease in the percentage and absolute numbers of polymorphonuclear leukocytes (PMN) (22,23). The responsible factor termed multiple sclerosis associated agent (MSAA) was found in brain, spinal fluid, serum and spleen of MS patients, but was absent in control samples. It appeared to be a small virus, which passed through 50 nm but not 25 nm millipore filters, and could be transmitted on second passage to other mice. Similar results have also been obtained in scrapie (24). The PMN

depression assay is subject to many variables and as these results have not been confirmed in tests on coded specimens, it is difficult at this time to assess their significance. Independent attempts to reproduce these results have been unsuccessful (25). An association between canine distemper virus (C.D.V.) and MS has been suggested as a result of the finding of an increased incidence of MS among house dog owners (26,27). In a survey of 60 MS patients and 40 controls the immunofluorescent IgG antibody titres to distemper virus in serum were significantly elevated ($P < 0.02$). More recently a cytopathic effect has been observed when bone marrow from 4 out of 5 MS patients with recent exacerbations was inoculated into cell cultures (28). The effect, which was not observed with bone marrow from controls, was inhibited by each MS patient's serum and not by the serum of 5 healthy subjects. The ether-lability of the isolates and the suggestion from neutralisation experiments of a possible antigenic relationship to C.D.V. and to a lesser extent to measles virus suggest that the agent might be related in some way to the paramyxoviruses. The possible involvement of paramyxoviruses as agents in MS has been critically discussed by Haire (16).

Summary

Lesions in the CNS show sites of predilection in areas in contact with the CSF and close to blood vessels. This suggests that a destructive factor of some kind may emanate from the blood stream. Active plaques show glial proliferation and many are close to perivascular cuffs of inflammatory cells. Oedema of the myelin sheath frequently appears as an early sign; intact myelin fragments are not usually observed close to areas of demyelination but there is a report of 'microglia' containing cytoplasmic inclusions consisting of membrane-bound stacks of curved linear profiles in MS plaques. In at least some chronic cases, there is a permanent population of plasma cells which may be responsible for the continuing synthesis of antibody. A large number of CNS lymphocytes are sequestered in recesses of the subarachnoid space over the surface of the cerebral hemispheres; antibody (IgG) is deposited in plaques and around astrocytic end feet. In apparently unaffected areas, there may be destruction of oligodendrocytes and proliferation of astrocytes. Although paramyxovirus-like inclusions have been seen in brain and in bone marrow tissue, these may turn out to be artifacts.

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Chapter 6

Biochemical Changes in the Central Nervous System of Multiple Sclerosis Patients

Histochemical examination of the central nervous system (CNS) in multiple sclerosis (MS) shows focal areas of demyelination in which sudanophilic lipid material accumulates with Marchi-positive staining. This has led investigators to examine the lipid composition of affected and normal tissue as has been successfully achieved for the leucodystrophies. The discovery of enzymatic 'markers' for different cell types has led to such indices being applied to the problem of identifying the cellular reaction in MS tissue.

Chemical Pathology of the Demyelinating Lesion

Lipid and protein analyses of MS plaques give a quantitative assessment of the myelin loss observed histologically. There is an increase in water content and an extensive loss of all three classes of lipids (Table 6.1) (1,2,3,4). As may be anticipated cerebroside, the characteristic myelin galactolipid, is depleted in the plaque, but unexpectedly there is not an accompanying loss of sulphatide. The reduced concentration of phosphatidal ethanolamine, the major myelin phospholipid, is balanced by increased amounts of lecithin. Appearance of cholesteryl esters is one of the hallmarks of demyelination for only traces of sterol esters are present in normal nervous tissue. In early, active plaques, the cholesterol loss is accounted for by ester accumulation. The ganglioside content of plaques is increased on a dry weight basis and decreased on a wet weight basis (5). Increased extracellular space accounts for the latter result. The increase in dry weight concentration is consistent with the assumption that astrocytic elements contain more ganglioside than does myelin. Plaque gangliosides show many changes from the normal white matter pattern. The major myelin ganglioside, G_{M1}, is decreased in plaques and there is complete absence of sialosyl galactosylceramide (G_7), the only ganglioside derived from cerebroside. This unusual ganglioside is unique to the human CNS and is concentrated in white matter and enriched in myelin.

The reduction in total protein content in the plaque is not as extensive as the lipid loss. Selective loss of basic protein with the appearance of high molecular weight peptides was demonstrated in lesions in both acute and chronic MS (Fig. 6.1). Proteolipid protein is relatively resistant to proteolytic digestion, but the higher molecular weight protein of the Wolfgram doublet (W2) is converted to the lower molecular weight form (W1) in the plaque (Table 6.1) (6). Myelin proteins are replaced by glial fibrillary acid protein, thus accounting for the small change in total protein (7).

Brain immunoglobulin G. About two-thirds of MS patients have an elevated cerebrospinal fluid (CSF) gamma globulin. Tourtellotte and Parker reported an investigation into the distribution of immunoglobulins in the brain of patients with MS (8). They found an elevated gamma globulin concentration in "normal" appearing white matter, grey matter and plaques. The highest levels were found close to the edge of plaques. Lower levels were found in the centre of plaques, in

TABLE 6.1 Protein and Lipid Content of White Matter from MS Autopsy Samples

Sample	Controls	MS	
	WM	NAWM*	Plaque
(mg/g wet wt)			
Water content	716	712	884
Total protein	96	92	82
Total lipid	168	165	20
(mg/100 mg protein)			
Basic protein	11.6	9.6	2.7
Proteolipid protein	17.2	15.8	13.8
Wolfgram protein W1	3.8	4.6	6.0
" " W2	3.0	2.7	0.3
(mg/100 mg dry wt)			
Total cholesterol	13.7	12.0	6.8
Cerebroside	12.4	11.2	5.6
Sulphatide	3.5	3.0	3.3
Total phospholipid	22.2	24.7	12.5
(% total phospholipid)			
Ethanolamine phospholipid	35	34	31
Lecithin	25	26	29
Sphingomyelin	16.6	16	16
Phosphatidyl inositol	2.7	3.0	2.6
Serine phospholipid	20	18	16
(NANA†/100 mg dry wt)			
Ganglioside µg lipid	82.9	74.3	129.3

* Normal-appearing white matter

† N-acetyl neuraminic acid

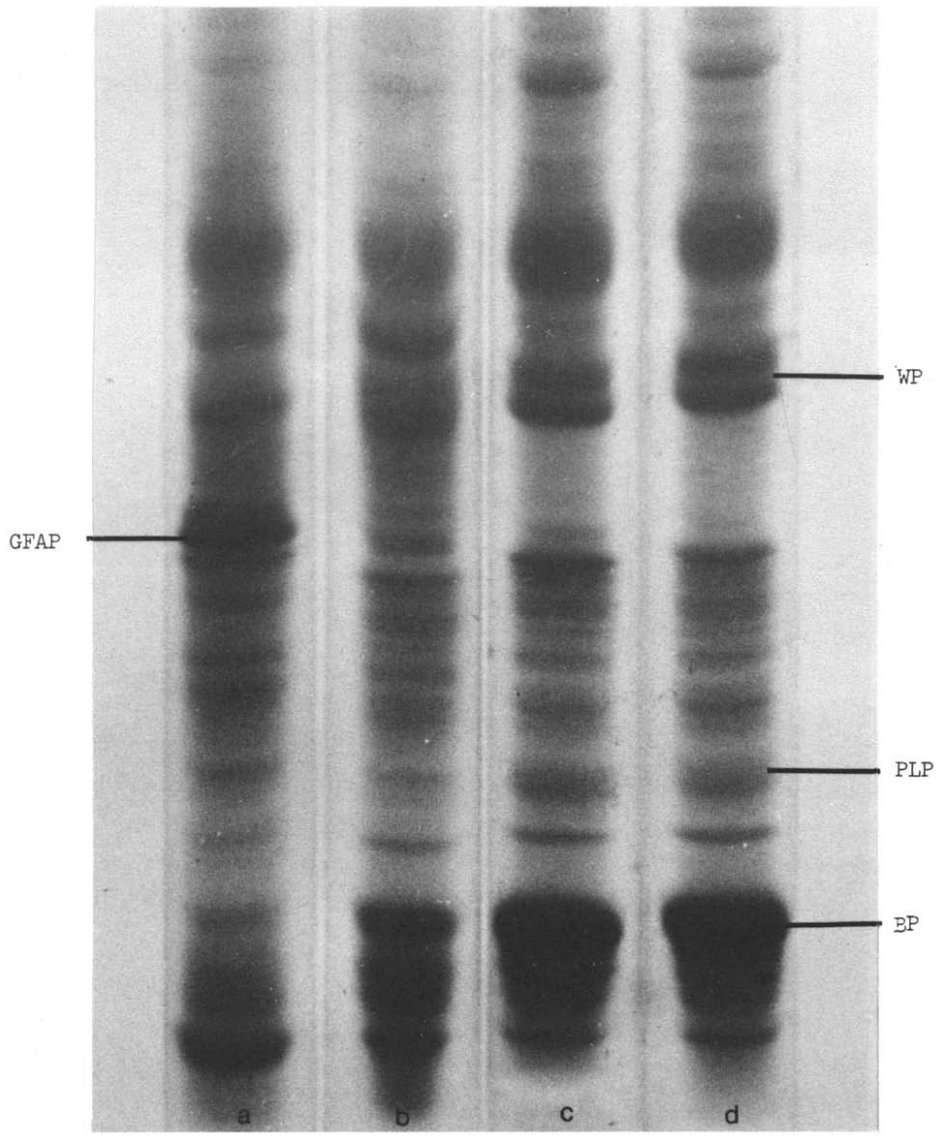

Fig. 6.1 Gel electrophoretic pattern of white matter proteins in MS. (a) MS-plaque, (b) MS-plaque rim, (c) MS-normal appearing WM, (d) control WM.

"normal" appearing white matter some distance from the plaques, and in normal appearing grey matter. The degree of perivascular accumulation of mononuclear cells correlated directly with the degree of elevation. This data supported their hypothesis that proliferation of perivascular cells was sufficient, and probably necessary, to produce an elevation of gamma globulin in the brain tissue in MS. But it was possible that cells in the advancing margin of a plaque might also produce gamma globulin. In a further paper, they suggested that the elevated gamma globulin in the "normal" white matter could be due to diffusion

of immunoglobulin synthesised in an active plaque. The increase in CSF globulin was likewise a reflection of excess globulin in the brain. The elevated globulin could be used as support of two theories about the aetiology of MS; namely that it is due to an autoimmune process or to a slow virus infection.

Myelin and Normal-Appearing White Matter

It has been reported from several laboratories that there are abnormalities in lipid composition of white matter and myelin in MS, which point to a primary lipid defect of myelin. Significantly lower levels were found for serine phosphoglycerides and sulphatides of both white matter and myelin by Clausen and Hansen (9), Woelk and Borri (10) and Alling and co-workers (11). These results do not support the earlier findings of Cumings and Goodwin (12), and Gerstl and co-workers (13), who found less cerebroside with a concomitant increase in sulphatide, in MS myelin. However in all 9 samples from 3 MS brains Gerstl and co-workers (13) found lower values for serine phosphoglycerides, but did not comment on this result. The fatty acid patterns of MS brain are strikingly similar to those of normal brains (11). In cerebrosides and sulphatides 24 h : 1 were significantly increased and 24 h : 0 decreased. The only phospholipid in MS brain with a significantly different fatty acid pattern from normal brain is sphingomyelin; the amount of very long-chain unsaturated fatty acids is reduced, particularly 25 : 1. On the other hand no abnormalities in lipid composition, including gangliosides were found in myelin isolated from white matter of 5 patients with MS by Suzuki and co-workers (14). The only change found was a statistically significant reduction of 25-30% in myelin yield.

Reports of changes in proteins of normal-appearing white matter from MS brains are few. The myelin fraction isolated by Suzuki and co-workers contained normal amounts of basic and proteolipid protein, a finding which confirmed earlier data. In addition Wolfgram (15) found a normal amino acid composition in MS myelin, suggesting a normal protein pattern. Even where an extensive diffuse form of demyelination was reflected in a very low recovery of myelin (16), the isolated fraction had no significant compositional abnormalities. As the chemical composition of the myelin sheath in MS white matter differs only slightly from that of control white matter, there is little evidence for a primary compositional defect. The depletion in acid phospholipids may relate to an early loss of basic protein from damaged tissue with which these acidic lipids interact. In the white matter of cases of progressive MS of short duration, we have found cholesteryl esters and loss of cerebroside, sulphatide and phospholipid from areas which appeared normal to the naked eye. The myelin fraction isolated from normal-appearing white matter from cases of acute MS is of unchanged composition but the yield of myelin is reduced. As a result of a change in density abnormal myelin may be lost at the preparative stage in differential centrifugation.

Fatty acid composition in apparently normal areas of MS white matter. In 1962, Baker and co-workers (17) extracted lecithin from white matter of MS cases in which recognizable plaque tissue was not visible to the eye. Analysis of the fatty acid composition showed that the proportion of saturated chains was increased and unsaturated chains decreased. Other workers have found differences with ranges from +2.3 to +6.1% for saturated and -0.2 to -8.3% for unsaturated fatty acids (18). The increases were found in palmitic (16:0) and decreases in palmitoleic (16:1), oleic (18:1) and arachidonic (20:4). Varying abnormal results have been found by other workers (19) although Alling and co-workers (11) found that the fatty acid patterns of the MS brains were strikingly similar to those of normal brains. Only minor changes were found in some white matter ester fatty acids.

Thompson and co-workers (20) found small but significant alterations, in the serum lipid fatty acids. In particular, they found a reduction in linoleate (18:2) concentration, but Love and co-workers (21) reported no difference in linoleate concentration of fasting blood lipids when other neurological cases were compared to MS sufferers. However they confirmed that there was a difference between patients and healthy controls. It has been suggested by Thompson (19) that these changes indicate a widespread abnormality in the handling of certain fatty acids in the body of patients with MS. As a result of this hypothesis, long-term trials of polyunsaturated fatty acid (PUFA) supplementation in patients have been undertaken, with moderate improvement in the length of relapse in patients with progressive disease (22). It is known that C18:2 and C20:4 are precursors of prostaglandins and hence reduced synthesis of prostaglandins may be a feature of the disease. Possibly PUFA act as immunosuppressants and their clinical effect is related to this property. Clausen and Møller (23) found an increased susceptibility to experimental allergic encephalomyelitis (EAE) and a more severe course of this disease in animals bred and raised on a PUFA diet. On the other hand dietary supplementation with PUFA exerts a protective effect (24).

Lysosomal Hydrolases

In MS it has been postulated that release of lysosomal enzyme activity is responsible for the initial attack on the myelin sheath. Measurement of hydrolase activity gives an indication of the possible mechanism of demyelination and the pattern of raised enzyme activity may be used to attempt to identify the cell type involved.

Chronic plaque. In established chronic lesions with minimal cell cuffing, the largest increase is in the activity of acid phosphatase, followed by acid proteinase, with minimal β-glucuronidase activation (25). In contrast, the activity of the myelin marker enzyme, 2',3'-cyclic nucleotide phosphohydrolase is dramatically decreased and there is a reduction in acid lipase - esterase and leucyl- β-naphthylamidase activity, suggesting that these enzymes are localized in myelin or oligodendroglial cells.

Active plaque. The early, active demyelinating plaque is marked by perivascular cuffing of lymphocytes with proliferating neurological cells and/or macrophages. The lysosomal hydrolase activity is concentrated at the rim of the plaque where there is evidence of increased metabolic activity (26). The centre of the plaque sometimes has hydrolase levels below those of unaffected white matter. In addition to increased acid proteinase and phosphatase activity (4), raised levels of β-glucuronidase, arylsulphatase, plasmalogenase and cathepsin A are found in active plaques (3,27,28). Neutral proteinase activity has also been reported to be increased at the plaque border (26). These enzymes are typically found in PMN leucocytes and some is present in myelin. The degree of activation is not so significant as that of acid proteinase.

In a recent study in our laboratory, both β-glucuronidase and acid proteinase activities were significantly raised in active lesions, where widespread diffuse demyelination was accompanied by the accumulation of both macrophages and astrocytes (16). In well circumscribed plaques, which were associated with a greater degree of lymphocytic cuffing of vessels, the increase in acid proteinase was not so marked. Macrophages have a much higher acid proteinase/β-glucuronidase ratio (12:1) than circulating lymphocytes (3:1). Thus the very high acid proteinase levels found in a fulminating lesion may reflect astrocytosis and macrophage accumulation while the relatively greater increase in β-glucuronidase activity in discrete plaques may be a result of perivascular infiltration of

lymphocytes. In the chronic burnt out plaques, the source of enzyme activity must be attributed to fibrous astrocytes. In the massive inflammatory lesions of a monkey with EAE, Hirsch and co-workers found that β-glucuronidase was the enzyme whose activity was most increased (29) (Tables 6.2 and 6.3).

TABLE 6.2 Lysosomal Enzyme Activity in MS Brain

Units of enzyme activity of control white matter = 100

	Plaque centre	Plaque border/ plaque including border	Adjacent white matter	Normal-appearing white matter
p-Nitrophenyl phosphatase	-	394[a]	138[a]	169[b] 91[a]
Acid proteinase	75[b]	417[a] 179[b] 238[c] 195[d]	178[a] 142[d]	128[a] 124[b] 124[c] 136[d]
Neutral proteinase	68[b]	128[b]	-	101[b]
β-Glucuronidase	-	190[a] 136[c] 250[c] } e	105[a]	111[a] 122[c] 204[c] } e
Amino peptide-dipeptidase	64[b]	200[a] 145[b]	93[a]	108[a] 95[b]
Acid lipase-esterase	-	77[a]	93[a]	96[a]

a - Hirsch and co-workers (29)

b - Arstila and co-workers(26)

c - Cuzner and Davison (3)

d - Einstein and co-workers (2)

e - β-Glucuronidase results of chronic and acute MS respectively

TABLE 6.3 Lysosomal Hydrolases in MS Brain

Units of enzyme activity of control white matter = 100

	Cathepsin A[a]	Arylsulphatase[b]	Plasmalogenase[c]	Phospholipase A_2[d]
Plaque	136[e]	121[e]	211	-
	255[f]	145[f]		
Normal-appearing white matter	100	108	-	150

a - Bowen and Davison (28)

b - Cuzner and Davison (3)

c - Ansell and Spanner (27)

d - Woelk and Peiler-Ichikawa (30)

e - plaques from chronic MS brains

f - plaques from acute MS brains

Plasmalogenase, like phospholipase A_2, hydrolyses an intact phospholipid to produce lysophospholipid, which is myelinolytic. The enzyme is almost exclusively located in the white matter and in a study of a single MS brain, the plasmalogenase activity of several plaques was double that of the normal-appearing white matter (27). The source of the increased plasmalogenase activity could equally well be infiltrating cells or activated glial cells. The carboxypeptidase, cathepsin A, which acts synergistically with cathepsin D, is enriched in macrophages (specific activity 30 times greater than in brain). In comparison to the cathepsin A activity of cerebral matter (1.14 units) in control subjects, the mean activity in plaques from 4 chronic and 3 acute cases of MS was elevated 37% and 155% respectively (28). Thus the finding of increased cathepsin A activity in MS plaques is consistent with the view that macrophages are at least one source of the increased hydrolase activity.

Normal-appearing white matter. There is no significant elevation of hydrolytic enzyme activity in the grossly normal white matter of chronic MS brain, with the possible exception of acid proteinase. Arstila and co-workers, Einstein and co-workers, and Hirsch and co-workers all found acid proteinase activity to be increased in a large proportion of MS white matter samples, in comparison to controls. In our study of apparently normal white matter from two acute cases of MS, the β-glucuronidase level was significantly increased, although the level of acid proteinase was unchanged. The formation of recognizable demyelinating lesions may be preceded by enhanced metabolic activity, arising either from glial cells or from perivascular lymphocytes. Phospholipase A_2, which also has a lysosomal localization has been observed to increase by approximately 50% in macroscopically normal white matter from MS brain, with an accompanying decrease in ethanolamine plasmalogen (30). The concentration of total ethanolamine phosphoglyceride was not altered. Thus, a special role for acid proteinase in

myelin destruction appears unlikely and the increases in all the lysosomal hydrolases may be a result rather than a cause of myelin breakdown.

Summary

It seems that changes found in white matter of apparently normal areas are due to minute areas of damage not visible to the naked eye. The isolated myelin from the CNS of MS patients has a composition similar to that of control samples. There is no support therefore for the idea of a compositional defect in myelin before the onset of the disease process. Chronic areas of demyelination show loss of myelin components from the affected white matter and accumulation of cholesteryl esters and glial fibrillar protein. Where there is acute damage lysosomal hydrolase activity is increased and there is selective loss of myelin basic protein with appearance of lower molecular weight peptides. This attack may be initiated by the action of neutral and possibly acid proteinase, phospholipase and plasmalogenase secreted by activated cells. It is not established if oligodendroglial cells are first affected or whether the myelin basic protein has to be exposed before proteolysis occurs.

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Chapter 7

Clinical Pathology of Multiple Sclerosis

Relatively few tests have proved of value in the routine investigation of patients with multiple sclerosis (MS). The most useful test measures the change in the immunoglobulin G (IgG) concentration in the CSF, which is associated with a significant alteration in the gold colloidal precipitation curve. Additional information can be obtained from neurophysiological and immunological procedures.

Neurophysiology

Neurophysiological techniques, such as the visual-evoked potential (VEP), can help in quantifying the degree of central nervous system (CNS) damage in a particular subject (1). The technique of VEP recording provides an estimate of the change in size of the afferent volley reaching the cortex and is of special value in acute optic neuritis. Normal impulse conduction may be completely blocked or continued transmission may be delayed and there may be inability to transmit faster trains of impulses. Conduction block and the ability of the fibre to transmit fast impulses are mostly affected by temperature change. Occipital responses to monocular stimulation with a reversing black and white checker-board have been successfully used (1). In longitudinal evaluation of patients remarkable recoveries in amplitude and peak latencies have been observed. This result raises the important possibility that remyelination of the damaged optic nerve fibres may occur in some patients.

As an alternative to the VEP, the auditory system may be examined by use of a standardized click stimulus. The auditory-evoked potential (AEP) is more complex and depends on the structures in the brain stem as well as the forebrain. A high proportion of patients with clinical evidence of brain-stem demyelination have abnormal auditory-evoked responses and 50% of those without any apparent such disturbances also have abnormalities, in spite of the rarity of clinically apparent deafness (2). It is believed that ultimately a series of evoked-potential data in a single patient will enable hidden plaques to be detected in those patients with clinically probable, or possible, MS and thus make the diagnosis definite.

Neuroelectric Blocking Factors

Some of the recoveries seen in patients may be due to reversible effects of serum factors. Sera from MS patients and also from animals with experimental allergic encephalomyelitis (EAE) have the ability to reversibly block extra-cellularly recorded, evoked electrical response of CNS tissue cultures (3). The effect which is distinct from the demyelinating factor, seems to be complement-dependent and could be the explanation of some of the transient neurological changes seen in MS. However, blocking factors are also found in the sera of some normal subjects. In a comprehensive review Seil (4) concludes that: "... while there is evidence from a number of laboratories for the existence of serum factors in human and animal species which depress electrical activity in in vitro

preparations, disagreement exists about the specificity of such factors for demyelinating disease. Sera from normal rats, rabbits and humans, as well as from those with demyelinating diseases have been shown to block post-synaptic responses in mammalian spinal cord, hippocampus and cerebral neocortex cultures." In a recent study, Schauf and co-workers (5) studied the neuroelectric blocking activity of sera in MS patients and controls including patients with strokes. They found a significantly increased blocking activity in patients within 6 weeks of an acute exacerbation (Table 7.1). The activity was complement-dependent and found in the serum IgG fraction but the responsible antibody was not directed against measles virus. Nevertheless the role of serum neuroelectrical blocking factors, specific or non-specific, in the pathogenesis of human or experimental demyelinating disease remains undefined (4).

TABLE 7.1 Neurological Blocking Potency with Clinical Findings in MS

Stimulated frog spinal cord ventral root preparations were used to monitor 25% serum in Ringer's solution. Activity in Ringer's solution = 1.

Patient	Average Ventral Root Response	No.
Stable for 3 years	0.89 ± 0.04	62
Acute (within 6 weeks of a new sign or worsening of signs)	0.59 ± 0.05	20
Controls*	1.25 ± 0.03	19

* Similar results were obtained for 42 age and sex matched controls and 22 stroke patients. [After Schauf and co-workers (5)].

Blood

Relatively few alterations are seen in the blood of MS patients. It has been reported that blood platelets from patients with MS are abnormally sticky and this adhesiveness is inversely related to serum cholesteryl linoleate levels. Changes in red blood cells of MS patients, in relation to disease activity, have also been found with an increased mean erythrocyte diameter and fragility (6). These findings have been related to possible defect in membrane lipid composition. Thus there are reports of a deficiency in serum polyunsaturated fatty acids (PUFA) of severely affected patients (Table 7.2) (7). However such changes are also found in patients with other neurological diseases and may therefore be associated with hospitalisation or with disablement (for discussion see Chapter 6).

Immunological tests. A number of tests to measure the cellular immune response in MS have been described. None has yet been adopted for routine diagnostic investigations. For example, response by leucocytes to the encephalitogenic myelin basic protein (BP), protein has been used as the basis of *in vitro* tests for MS. The response seems to vary during the course of the disease. Källen and

TABLE 7.2 Percentages of Linoleic Acid (18:2) in the Total Lipid Extract of Serum of Fasting Subjects

After Thompson (7)			After Love and co-workers (8)		
Subjects	No. of Subjects	18:2 as % of total fatty acids (± SEM)	Subjects	No. of Subjects	18:2 as % of total fatty acids (± SEM)
Controls	(38)	25.6 ± 0.5	Controls	(49)	27.0 ± 0.9
MS	(47)	22.3 ± 0.5	MS	(47)	22.3 ± 0.7
No clinical deterioration over preceding months	(10)	25.8 ± 0.9	Neurological controls	(29)	20.9 ± 0.8
Worsening signs:-	(11)	23.6 ± 0.6	Acute illness	(35)	14.4 ± 1.1
	(16)	21.6 ± 0.6			
↓	(10)	18.6 ± 0.4			

co-workers (9) used the technique of leucocyte migration in agarose on MS patients to detect changes in reactivity to BP in connection with a relapse. Six out of ten of the patients showed significant reactivity within a few days after the relapse. This response decreased or disappeared during the two weeks after the relapse, but sometimes reappeared and was found in tests performed 2-3 months later. It was proposed that reactivity is an epiphenomenon due to CNS tissue destruction for similar results were obtained in some patients with cerebral infarction.

Levy and co-workers (10) have described a blood test based on the adherence of peripheral blood lymphocytes to measles virus-infected tissue culture. Purified populations of lymphocytes from patients with MS form rosettes *in vitro* when mixed with measles virus-infected epithelial cells. Similarly prepared lymphocytes from control populations showed much less rosetting activity; there was no overlap of patient and control values, suggesting that the assay may be of diagnostic importance. The positivity of the test in patients with MS was not affected by the severity, duration or activity of the disease. This is a difficult procedure and a wide range of further controls needs to be done before the test can be accepted as a routine procedure. There are conflicting results on specific cell-mediated immunity against measles virus (see Chapter 8) and with non-specific cell-mediated immune reactions *in vitro*. In one recent study, Nordal and Frøland (11) studied two patient groups, one with recent (but later than 3 weeks after

onset) and another with long standing disease. They found no significant difference in lymphocyte transformation reaction to a number of mitogens (excluding BP and measles), in rosette formation or in cytotoxicity.

The macrophage electrophoretic mobility test (MEM) was introduced by Field and Caspary (12) for the detection of malignancy. The test is based on the observation that peripheral blood lymphocytes from patients with malignant diseases are sensitized to BP or to a purified protein derivative (PPD). Incubation of sensitezed lymphocytes with either of these antigens resulted in the release of a macrophage-slowing factor from the lymphocytes, whose presence may be detected by its effect on the electrophoretic mobility of guinea pig macrophages. Peripheral blood lymphocytes from patients with MS appeared to be much more susceptible to the inhibitory activity of linoleic acid when tested for sensitization to PPD by the MEM test than are those from normal subjects (13,14). These differences were claimed to be specific for MS and could be used as an in vitro diagnostic test for the disease. However, there have been conflicting reports on the value of the MEM test, both in the diagnosis of cancer (15,16) and in the diagnosis of MS (17). A diagnostic test for MS with human red blood cells was then developed (18). In this test, it was claimed that erythrocytes from patients with MS showed a significant reduction in their electrophoretic mobility in the presence of linoleic acid, whereas erythrocytes from other patients or normal subjects showed an increase in their mobility. The effect of linoleic acid on the electrophoretic mobility of red blood cells from both MS patients and normal subjects was studied by Stoof and co-workers (19). An extensive statistical evaluation of the data clearly demonstrated that there was no difference in behaviour of red blood cells from patients and normal subjects in the presence of linoleic acid. Even a tendency for the mobility of erythrocytes to be decreased in patients and increased in normal subjects after addition of linoleic acid was not observable. Similar observations were reported by Forrester and Smith (20) who found no change in erythrocyte mobility with linoleic acid.

The Cerebrospinal Fluid in Multiple Sclerosis

Although the total mean protein content (about 40 mg/100 ml) of CSF is 200 times less than that of serum, the level of IgG is disproportionately lower (15% of the total protein in serum and 3% in CSF). In MS the concentration of total protein rarely exceeds 100 mg/100 ml but about 75% of MS patients have an increased concentration of IgG in the CSF (above 12% of total protein) and this increase persists throughout their illness. Since serum proteins are able to leak into spinal fluid as a result of damage to capillaries (e.g. plaques near the ventricles), corrrection must be made for the possibility of any such passive transfer by relating IgG values to another high molecular weight serum protein, e.g. macroglobulin or albumin. Thus Tourtellotte (21) corrects for a contribution from serum antibody by utilising the relative CSF and serum IgG and albumin concentration and applying these values in a correction formula. Olsson and Pettersson (22) advocate use of an IgG index (CSF/serum IgG ratio divided by CSF/serum albumin ratio). An increased index is found in 88% of MS patients and in only 18% of those with other neurological disease. Having allowed for this, MS is clearly still a disease in which the amount of IgG in the CSF is much higher than can be explained by simple 'transfer' of antibody from serum. This was also concluded by Frick and Scheid-Seydel (23) who found on the basis of exchange of intravenous ^{131}I-labelled IgG between serum and CSF, that immunoglobulins were synthesized in the brain of MS patients.

In addition Cohen and Bannister (24) demonstrated that CSF lymphocytes from an MS patient synthesized IgG and IgA but not IgM. The newly formed IgG had the same relative amounts of kappa and lambda determinants as the IgG present in the

CSF. These findings were confirmed by Sandberg-Wollheim and co-workers (25) who found that IgG and IgA were synthesized by CSF lymphocytes from MS patients particularly during a relapse. The synthesized IgG had an oligoclonal distribution and showed the same electrophoretic pattern as the IgG of the original CSF (26). Blood lymphocytes from the same patients synthesized an IgG in vitro that showed a completely different electrophoretic pattern. Furthermore, the amount of IgG synthesized by blood lymphocytes was less than the amount synthesized by the CSF cells. These results demonstrate that at least part of the oligoclonal IgG of the CSF of MS patients is synthesized intrathecally and suggest that the CSF cells are antigenically stimulated within the CNS in vivo. From the 500 ml of CSF formed and absorbed daily in a normal adult (21) it appears that the average MS patients' CNS produces an excess of about 16 mg of IgG per day (range 9-100 mg). This synthesis is depressed during steroid therapy in parallel with clinical improvement.

Oligoclonal bands. In the normal subject the IgG region of electrophoretically separated CSF has a diffuse homogenous appearance but in MS and in diseases of the nervous system where there is a known 'infectious aetiology' (e.g. mumps and herpes encephalitis, multifocal leukoencephalopathy or in sarcoid) a restricted heterogeneity of the IgG is seen. These oligoclonal bands of antibody (27,28,29, 30,31) are relatively diffuse in themselves and probably represent the immunodominant antigens, each of which has stimulated several clones of lymphocytes to produce antibody. In some 90% of MS patients oligoclonal bands can be detected (29,32). In addition, in about 53% of patients (33) there is an alteration in the ratio of kappa:lambda light chains suggesting some abnormality in the nature of the IgG molecule. The kappa type proteins are more pronounced in MS and in conditions of chronic antigenic stimuli, e.g. herpes and measles. Free light chains, identified in the CSF of patients with subacute sclerosis panencephalitis (SSPE), were found in only a few MS patients (34). These free light chains may be

TABLE 7.3 Concentration of IgG & Kappa : Lambda Chain Ratio in CSF & Serum

	CSF				SERUM			
	IgG		kappa:lambda chain ratio		IgG		kappa:lambda chain ratio	
	median ($\times 10^3$)	range ($\times 10^3$)	median	range	median	range	median	range
Normal cases (n=9)	1.7	1.4-2.8	1.1	0.9-1.5	1.0	0.9-1.4	1.0	0.8-1.4
MS with IgG bands (n=11)	8.7	2.8-12.0	2.6	0.9-5.6	0.9	0.7-1.5	1.1	0.9-2.0
MS without IgG bands (n=3)	1.8	1.5-2.2	0.6	0.6-0.6	0.8	0.8-0.9	0.9	0.6-1.3
Other neurological disorders (n=22)	4.8	2.1-23.0	1.1	0.8-1.7	1.0	0.8-1.6	1.1	0.7-1.6

After Link and Zettervall (33)

derived from proteolysis of oligoclonal immunoglobulins synthesized within the CNS or there may be a defect in assembly of the complete antibody molecule. No change was seen in the γ-globulin CSF pattern during a longitudinal study of the CSF of 4 MS patients by Vandvik (35), but a recent report suggests that the oligoclonal band pattern does alter during the course of the illness (36).

Nature of the antibody and its specificity. The IgG isolated from both nervous tissue and CSF from MS and SSPE patients is mainly of the IgG_1 subclass (37). Oligoclonal measles-specific antibody can be isolated from sera, CSF and brain antibody in cases of SSPE and to a lesser extent from MS patients (38). In the latter case, the antibody eluted (about 20 mg IgG) from the serum and brain plaque tissue contained several bands of IgG. All 4 IgG subclasses were detected in the serum whereas only IgG_1 was found in the plaque. Using haemagglutination-inhibition tests and immuno-electrophoresis against unpurified cell-associated virus, the antigen cross-reaction was primarily with the IgG_1 subclass. However, measles virus antigen has not been identified in MS plaques, choroid plexus or in lymphocytes (39) and oligoclonal bands in the CSF are not absorbed out by measles virus antigen. When concentrated samples of CSF are analyzed, the IgG_2 and IgG_3 subclass proteins are found (40). IgG_4 can only be quantitated in the CSF of individuals having a relatively high serum IgG_4 content. Using specific antisera for each subclass it has been shown that all IgG subclasses are present in the CSF of MS patients and 'normal' individuals. Although the total IgG content of the CSF of MS patients is raised compared to the values obtained for the CSF from 'normals' this is not usually reflected in increased levels of IgG_3 and IgG_4. The increased IgG levels are predominantly reflected in increases in the IgG_1 subclass level. It appears likely that the observed increased IgG_1 levels are indicative of a selective local stimulation of antibody synthesis rather than selective transport across the blood brain barrier. CSF antibody directed against a variety of other paramyxovirus has been detected but these antibodies account for only a small proportion of the total and frequently are seen in other neurological diseases (41). CSF complement-fixing antibody reacting with crude MS brain has been detected by Laurell and Link (42) and by Ryberg (43), but the proportion of such antibodies has not been established. Finer analysis of the antibody spectrum of CSF oligoclonal bands has been achieved by electrofocusing (Fig. 7.1) (44).

Cells. In about 66% of patients in remission or relapse, the total leucocyte count in the CSF is normal (i.e. less than 6 leucocytes/mm^3). In 95% of patients, the count does not exceed 15/mm^3. Polymorphonuclear leucocytes (PMNL) in the CSF are rarely seen in MS (less than 0.2% PMNL) however, Tourtellotte (21) has reported 2 cases of MS with 19%and 25% PMNL), and Whitaker (45) finds PMNL in 7/14 acute cases of MS (Table 7.4). In some MS cases the number of plasma cells may be increased. There have been reports (46,47) of an increase in T lymphocytes in the CSF during an acute relapse,and macrophages may be present (see Chapter 8). Similarly, Allen and co-workers (48) found that during a relapse the percentage of T cells increased (65%), diminishing to 41% in the second week after an attack and reaching 30% in remission. These findings contrast with the variations found in the peripheral blood T and B cell populations of MS patients (49,50). There are reports of an increase in B (thymic-independent) lymphocytes in subjects with MS and a decrease in T (thymic-dependent) lymphocytes in the blood of patients with acute MS.

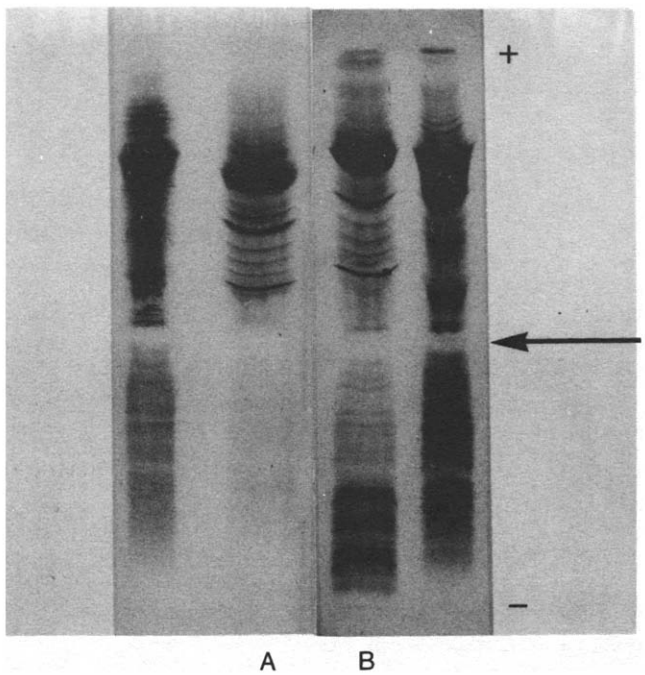

Fig. 7.1 Isoelectric focusing of CSF (44). The normal IgG region extends from the sample application (arrow) to the cathodal end. A: normal CSF. Serum sample on the left. B: MS CSF. Serum sample to the right. Increased concentration of CSF IgG, especially those of alkaline pI values.
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Summary

Apart from neurophysiological examination and study of CSF antibodies, few other investigations are of routine value in MS. Tests of lymphocyte function and macrophage electrophoretic mobility are still controversial although some of these investigations are suggestive of an abnormal cellular immune response. In the serum, a complement-dependent neuroelectric blocking antibody has been found during a relapse; this may account for some of the reversible clinical signs occurring during an exacerbation. Examination of the CSF proteins and especially electrophoretic separation of oligoclonal bands has been of considerable diagnostic value. It seems clear that much of the antibody is synthesized within the CNS. There is an important report that the oligoclonal band pattern alters during the course of the illness. Occasional polymorphs and some active T cells appear in the CSF during an exacerbation.

TABLE 7.4 Human IgG Subclass Proteins in Controls CSF and MS Sera

	Typical Analysis			
	Control		MS	
	Serum g/l	CSF g/l x 10^2	Serum g/l	CSF g/l x 10^2
IgG_1	6.8	4.6	6.8	6.4
IgG_2	2.7	2.4	4.3	5.0
IgG_3	0.9	0.3	1.2	0.3
IgG_4	0.7	Trace	1.4	0.5

Probable role of the IgG subclasses:-

Percentage of Total Serum IgG	IgG_1 60	IgG_2 30	IgG_3 5	IgG_4 4
Complement activation by Clq binding	++	(+)	+++	-
Alternative pathway complement activation	+	+	+	-
Binding to mononuclear cells	+	-	+	-
Placental transfer	+	+	+	+
Reactivity with staphylococcal protein A	+	+	-	+
Skin sensitizing activity for passive cutaneous anaphylaxis	+	-	+	-
Rheumatoid factor binding	+	+	+	+

After Jefferis and co-workers (40)

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Chapter 8

Immunology

Both humoral and cellular factors have been implicated in the pathogenesis of multiple sclerosis (MS). Lymphocytes are found close to demyelinating lesions, and there are changes in T and B cell populations as well as raised antibody in the cerebrospinal fluid (CSF) of most MS patients.

Humoral Factors

Small changes in the total serum antibody concentration in patients with MS have been reported, but it has not been possible to ascribe these elevated levels directly to MS (1). Virus-specific antibodies have been detected both in serum and CSF (2). For example, increased titres have been reported for antibody to different myxoviruses (e.g. measles, herpes, parainfluenza and rubella), but these account for only a small proportion of the IgG in the CSF and serum. A recent preliminary finding (3) has been the antibody directed against oligodendroglia found in sera of 19 out of 21 MS patients. The antibody was present in 3 out of 5 cases of subacute sclerosing panencephalitis (SSPE), but not in patients with other neurological diseases or in normal controls. Abramsky and co-workers (3) used an indirect immunofluorescence technique for detecting the antibody binding to isolated bovine oligodendrocytes or to human brain sections. The antibodies could be removed by pre-incubation with isolated oligodendrocytes or whole white matter, but not myelin or non-nervous tissue. Similar immunofluorescent staining was seen with rabbit anti-oligodendrocyte serum and the same serum could block the reaction with MS serum antibody. Antibodies directed against the encephalitogenic basic protein have not been found in the serum of MS patients, but they may appear in the CSF at the time of an exacerbation (4).

Myelinolytic and gliotoxic factors in the serum. In 1961 Bornstein and Appel (5) observed that the serum of rabbits with experimental allergic encephalomyelitis (EAE) caused demyelination of cerebellar explants in tissue culture. Later it was found that the serum of some MS patients and that of a smaller number of control subjects contained a similar factor. The demyelinating factor is found in about 60% of patients with active MS, in 30% where the disease status is less sure, and in 10% of those in remission. It is present in 10% of controls (6) and in 50% of cases of motor neurone disease (7). The factor has been reported as present in IgM and IgG fractions of sera of patients with active MS (8). Serum antimyelin factors are not induced by the encephalitogenic agent myelin basic protein and anti-basic protein antibodies do not have antimyelin effects in vitro. The myelin component(s) that induce(s) the factors has not yet been identified, although galactocerebrosides have been shown to raise them in rabbits (9).

Wolfgram and Duquette (10) made the important observation that the myelinotoxic action of MS sera could be absorbed out by a non-myelin white matter membrane fraction (this includes glial membrane as well as intracellular membranes). The demyelinating action was not removed by incubation with purified myelin although in contrast myelin neutralizes the demyelinating activity of EAE sera. Similar fractions from peripheral nerve were not effective in neutralizing myelinolytic activity. Wolfgram and Duquette concluded that 'if antibodies are involved in

the demyelination in MS patients, they are probably anti-oligodendrocyte rather than anti-myelin'. Despite the similarity of the phenomena in human and experimental conditions, the results would suggest that the demyelinating factor in MS is induced by a different antigen than that in EAE and may have a different meaning with regard to pathogenesis. It also seems probable (11) that the inhibitory action of serum from an acute MS patient on incorporation of ^{3}H-lysine into myelin protein by rat brain slices can be attributed to an action on glial myelin synthesis in vitro. Studies by several groups have shown that MS sera is toxic to glial cell cultures (12,13), but there are some doubts about the use of gliotoxicity as the sole index of a presumably disease-related effect (14). Raine and co-workers (15) studied the primary demyelination of organotypical cultures of mouse spinal cord produced by myelinotoxic MS sera. They showed myelin degeneration with transformation of the regular 12 nm periodicity into smudged amorphous structures. Eventually the degenerating myelin was phagocytosed by the investing astroglial cell cytoplasm until only axons, surrounded by astrocytic whorls, remained. There was growth of oedematous astrocytes, and oligodendrocytes appeared to be undergoing degeneration. Affected oligodendrocytes were phagocytosed following their ensheathment by astrocytic processes. Neurons and astrocytes did not degenerate.

Immune complexes. Reaction of antibody and antigen resulting in soluble immune complex formation is thought to result from continued antigenic stimulation. A raised IgG concentration in the CSF of the majority of MS patients is a consistent feature of the disease. In addition there is good evidence of serum immune complex formation in MS. With the Raji cell assay, circulating immune complexes have been detected in 49% of sera from MS cases and in only 15% of sera from normal controls (16). Circulating immune complexes have also been found to be raised in 29% of MS patients by Jacque and co-workers (17) and in 65% of MS patients by Goust and co-workers (18). In a longitudinal trial, we have found that a larger proportion of patients with MS and optic neuritis have slightly elevated levels of serum immune complex compared to normal and neurological controls, but this difference did not reach statistical significance (19). No correlation was found between the clinical status and the complex level in a longitudinal study of five patients. In this study the method of Nydegger and co-workers (20) was used; this measures complexed ^{125}I-labelled Clq (a subfraction of the first component of complement which initiates complement activation by binding to complexed immunoglobulin). As the Raji cell receptors are not specific for complexes bound to Clq only, this may explain the discrepancy between these results and the highly significant results of Tachovsky and co-workers (16). There is some data to suggest deposition of complexes within the brain of MS patients. An increased concentration of IgG has been demonstrated in plaque material from MS brains (21). Immunofluorescence and immunoperoxidase studies carried out on MS plaques have revealed the presence of myelin-bound IgG, as well as lymphoid and astrocytic cells with IgG-rich cytoplasm. Granular deposits of Clq in the same locations as IgG lend support to the view that the IgG found in the MS plaque is part of an immune complex in aggregated form (22). Where there is such deposition of immune complexes an inflammatory reaction may result.

The Inflammatory Reaction

The deposition of antigen-antibody immune complexes [for review, see Movat (23)] is associated with an inflammatory reaction ranging from an increased vascular permeability to severe haemorrhagic and necrotizing lesions. In the presence of excess circulating antigen soluble immune complexes are formed (e.g. in serum sickness) which are not removed by the reticuloendothelial system. Primarily arteritis, glomerulonephritis and arthritis develop as the small size of the

complexes thus formed favours deposition in tissues. Antibody produced is mainly IgG and IgM but some IgE is also found. The interaction of the latter (fixed to mast cells and basophils) with antigen leads to release of vasoactive amines. Complexes appear to be trapped in specialized filtration structures (e.g. renal glomerulus, choroid plexus) which are permeable to serum proteins. Deposition of circulating complexes requires increased vascular permeability probably mediated through vasoactive amines largely derived from platelets. In the presence of high circulating antibody, introduction of a small amount of antigen induces an Arthus reaction which is characterized by hyperaemia, exudative swelling, haemorrhage and necrosis. In addition to immune complexes complement and polymorphonuclear leucocytes (PMNL) are necessary for the inflammatory reaction. The PMNL phagocytose the deposited immune complex and become degranulated, releasing their lysosomal enzymes into the surrounding tissues.

PMNL and antigen-antibody induced tissue injury. PMNL and macrophages can exert deleterious effects in experimental inflammatory diseases (e.g. acute glomerulonephritis, arthritis, vasculitis). Thus the Arthus vasculitis can be inhibited by specific removal of neutrophils and in rabbits with serum sickness the usual necrotizing arteritis does not appear if neutrophils are removed. When antigen-antibody complexes are phagocytosed by PMNL, a number of phlogistic substances are secreted, including proteases. Acid proteinase can degrade basement membrane and a battery of neutral proteinases are secreted, including elastase, collagenase, cathepsin G and lysozyme. PMNL constituents are thought to be involved in the activation of thrombin and complement, possibly at Cl and C_5. Cationic proteins present in PMNL have bactericidal properties but they are also vasoactive and increase vascular permeability. Immune complexes also induce selective release of acid hydrolases from macrophages and in chronic inflammation plasminogen activator and lysozyme are actively secreted (24).

The inflammatory response in blood and CSF. Reports of increased PMNL neutral proteinase (NP) in the blood of patients with MS who are in relapse (19) point to an altered response of phagocytic cells of the immune system at this time. As well as small changes in total activity, the distribution of the enzyme was shifted from the granule to the cytoplasmic fraction (25). NP was significantly elevated and acid proteinase (AP) lowered in patients with clinically definite MS who had suffered a relapse within the preceding month. There was no correlation between enzyme activity and the lesion site or degree of diability (Fig. 8.1). Despite the difficulty of establishing an exact time relationship, in a longitudinal anaylsis of two cases, the rise in enzyme activity appeared to be preceded by a clinical relapse. The NP activity is predominantly located in the PMNL and the fluctuation of neutral and acid proteinase activity would not appear to be directly related to disease activity in the CNS itself but to relate to an extracerebral immune reaction, which could alter the functional properties of the PMNL. The alteration in NP activity suggests that an inflammatory reaction accompanies a relapse in MS. PMNL only appear rarely in the CSF during an exacerbation of MS but using a highly sensitive method with radioactive basic protein as substrate it is possible to separate cells (probably macrophages or polymorphs) containing NP. Little NP activity was found in lymphocytes from the CSF; in comparison, NP activity of PMNL in infectious diseases of the CNS was proportional to the cell count. NP activity of the CSF cellular fraction was significantly increased in MS patients in relapse compared with those in remission although PMNL and macrophages were not identified in the CSF even in the low number ($1\text{-}2/mm^3$) which would account for the observed NP activity. The presence of increased lysozyme levels (26) (known to be secreted by PMNL and macrophages) and the appearance of myelin fragments in the CSF of MS patients during an exacerbation are also indicative of inflammatory demyelination. Cohen and coworkers (27) and Whitaker (28) have reported an increase in basic protein or its fragments in the CSF which correlates with the clinical stage of the disease.

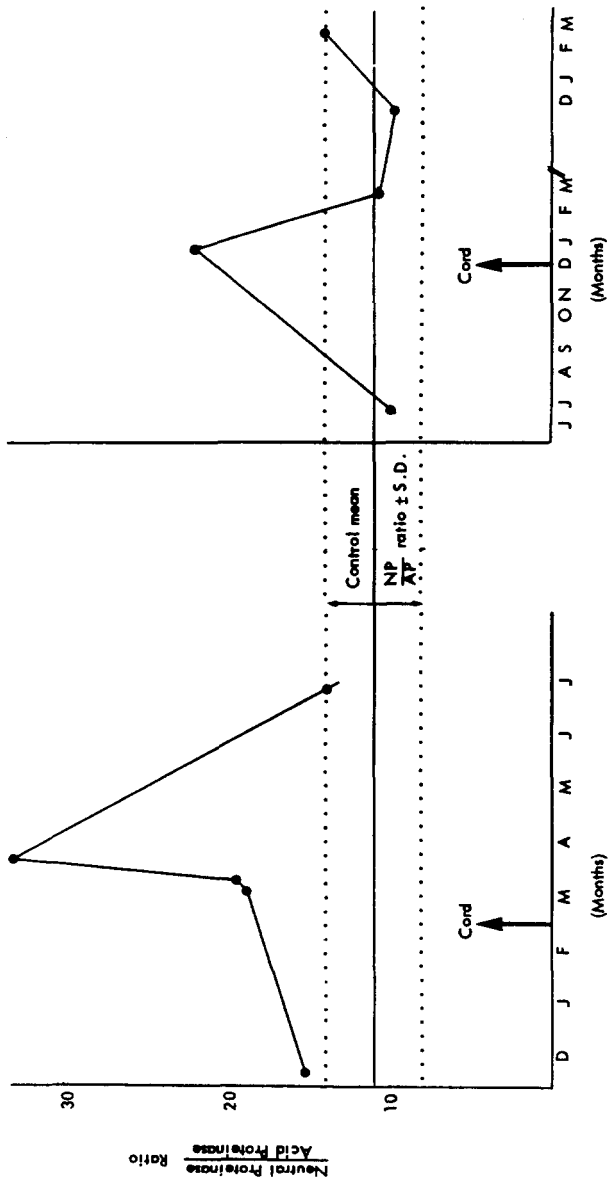

Fig. 8.1 Leucocyte $\frac{NP}{AP}$ ratio. Longitudinal analyses of two MS patients, showing the relationship between enzyme activity and a single relapse. Reproduced with permission of Ann. Neurol.

Using a double antibody radioimmunoassay technique, the CSF of 8 out of 14 MS patients in an acute phase of the disease had levels of 3.4-15.4 mg/ml of the P1 fragment (residues 43-88) of myelin basic protein (28). The fragment was absent in 29 patients with stable disease. The pleocytosis noted in many of the acute phase patients was predominantly mononuclear but in half of the CSF's PMNL were also present (Table 8.1).

TABLE 8.1 Clinical Data and CSF Findings in 14 Patients with MS in the Acute Phases of Disease

Time since onset of exacerbation (days)	Protein (mg/100 ml)	IgG (%)	Cells (per mm^3)		Cells (% of WBC)		Basic Protein (43-88) ng/ml
			RBC	WBC	PMN	Mono	
2	96	18	0	28	40	60	15.4
3	96	18	315	43	24	76	10.2
6	81	11	11	2	0	100	4.4
5	44	23	0	49	1	100	6.4
10	40	38	0	7	0	100	3.4
1	69	16	0	32	19	81	7.4
1	55	14	0	4	0	100	12.8
14	57	18	0	7	15	85	4.8
2	61	17	675	0	0	0	0
8	48	31	322	2	0	100	0
8	50	12	0	1	0	100	0
9	51	10	0	52	0	100	0
10	57	25	44	14	20	80	0
8	96	19	60	45	4	96	0

After Whitaker (28)

Lymphokines and prostaglandins. Although both lymphocytes and macrophages have been shown to be capable of direct cell-mediated cytotoxicity, chemical mediators are also involved: lymphokines, lysosomal enzymes and prostaglandins (29). Lymphokines are a mixture of soluble, highly biologically active factors secreted by stimulated lymphocytes. They are critical to the delayed hypersensitivity reaction e.g. increasing vascular permeability in vivo, ihibiting macrophage and PMNL migration, as well as stimulating macrophages. There is increased formation and secretion of lysosomal enzymes, which damage tissue in chronic inflammation. Prostaglandins, particularly of the E series (PGE_1), are secreted by macrophages and potentiate the inflammatory response of delayed hypersensitivity. But PGE_1

is also an effective inhibitor of PHA-induced lymphocyte activation (30), and impairs lymphokine production or secretion (31) from sensitized lymphocytes by a feed-back control system. Defective sensitivity of the small lymphocyte to prostaglandins may be one way in which chronicity persists. Inhibition of prostaglandin synthesis by non-steroidal anti-inflammatory drugs (such as Indomethacin) may not necessarily be acting at the most effective control point for the less active PG-synthetase inhibitor, acetylsalicylic acid, is a relatively successful drug in the treatment of the chronic disease, rheumatoid arthritis. Thus PG analogues may have a potential use in controlling chronic inflammatory conditions, such as MS.

Cellular Immunity

The finding of lymphocytes in the perivascular cuff close to lesions in MS and the alterations reported in the properties of circulating cells support the view that a delayed hypersensitivity reaction to viruses or other antigens could operate in MS. Offner and co-workers (32) confirmed the observations of Zabriskie and co-workers (33) of cellular anergy to measles antigen. Both radioactive thymidine and myo-inositol incorporation into MS lymphocytes was decreased on stimulation by PHA and measles antigenic components. This impaired lymphocyte function suggests a possible failure on the part of the lymphocyte to identify an antigen such as a virus. A defect of this type may be related to inherited factors as indicated by the Dw3-HLA determinants (see Chapter 1). On the other hand a positive correlation has been found for cellular hypersensitivity to myelin basic protein (BP) in relation to clinical attacks of MS (34). The results of both lymphoblastic transformation and macrophage migration inhibitory factor (MIF) assays relate to the temporal course of MS. In the MIF assay normal control subjects gave a mean value of 100, whereas patients studied within 4 weeks of onset of illness gave a result of 59. A convalescent and chronic group gave means of 86 and 91 respectively. Different portions of the BP molecule may stimulate sensitized lymphocytes to produce specific lymphokines. For example, a positive lymphoblast transformation result contrasted with a negative result for MIF, when MS lymphocytes were stimulated by the 17 amino acid C-terminal fragment of BP (35). Cellular hypersensitivity to brain fractions other than myelin proteins have been reported by Alvord and co-workers (36) and Offner and co-workers (37).

More definite changes in T cell activity have been found in the CSF where a small number of lymphocytes appear during a relapse (Table 8.2). In the CSF, Allen and co-workers (38) found that during a relapse the percentage of T cells in the first week increased (65%), diminishing to 41% in the second week after an attack and reaching 30% in remission. This direct variation in T cells accompanied by an increase in unreactive cells further implicates cellular immune mechanisms in the pathogenesis of MS. These workers suggest that the increasing proportion of unreactive cells in the CSF may be suppressed or tolerant myelinotoxic cells inactivated with a coating of antigen or antigen-antibody complex. In a prospective study of patients, Sandberg-Wollheim and Turesson (39) found an increased number of IgG-positive cells in the CSF, when compared with blood, and proportionately more T cells and fewer B cells. The significance of both these studies is subject to the limitations of techniques. It has not yet been possible to determine the percentage of T and B cells in normal CSF. But the percentage of B and T cells in peripheral blood is well-documented and a decrease in the relative and absolute number of T cells in the blood of MS patients has been reported by several groups (40,41). These results however show no correlation with clinical activity of the disease.

TABLE 8.2 Percentage of CSF T and B Cells at Different Times after Onset of New Symptoms of MS

Times after onset	No. of assays	T	B	Null	Total
1 week	7	7.4 (65.4%)	1.3 (9.2%)	2.7 (28.5%)	11.4
2 weeks	10	7.6 (40.7%)	1.6 (8.6%)	9.5 (51.5%)	18.7
3 weeks	7	5.9 (43.9%)	1.7 (20.1%)	3.4 (36%)	11.0
remission	10	0.9 (29.6%)	0.3 (7.0%)	1.8 (66.2%)	3.0

Total T cells were estimated by E-rosette assay using sheep erythrocytes. (After Allen and co-workers, 38).

Increased activity of sensitized lymphocytes (both T and B cells) may be induced by exposure to a suitable antigen. The findings suggest that cellular immune reactivity to BP occurs in the CNS in MS and this relates to clinical activity in the disease. Lisak and Zweiman (42) measured the lymphocyte reactivity to BP by the incorporation of labelled thymidine into incubated cells. When data from stimulated peripheral blood lymphocytes is compared to that for CSF-lymphocytes (Table 8.3), it is possible to correct for variability in response of lymphocytes to BP. Increased thymidine uptake occurred repeatedly in CSF lymphocytes cultured with BP at a time when blood lymphocytes were non-reactive to this

TABLE 8.3 Response of Cultured CSF and Blood Lymphocytes to Myelin BP in MS and other Neurological Diseases †

		Unstimulated CSF lymphocyte culture	Stimulated to BP	Mean of differences CSF/blood*
Acute MS	(5)	990 ± 576	1896 ± 783	1518 ± 700
Progressive MS	(5)	312 ± 243	1250 ± 740	802 ± 820
Stable MS	(9)	105 ± 37	170 ± 94	-200 ± 369
Acute disseminated encephalomyelitis	(6)	441 ± 232	956 ± 213	-236 ± 610
Other neurological diseases	(9)	326 ± 161	88 ± 79	-869 ± 689

† Mean (cpm ± SEM) per 5000 cells.

*Mean differences in response between CSF and blood lymphocytes for subjects in each clinical group (cpm ± SEM). A positive value indicates overall greater responsiveness in CSF for that patient [Lisak and Zweiman (42)].

antigen in vitro. This contrasting pattern of reactivity was seen most commonly in cells obtained close to the time of acute exacerbation, to a lesser extent during the progressive stage of the disease and not at all during the remission period. There is increased uptake of thymidine in untreated CSF lymphocytes, suggesting a possible in vivo stimulatory effect during the course of the disease. This reactivity of the lymphocyte might be accounted for by endogenous stimulation of long-surviving lymphocytes or reflect entry of monocytes from the blood. It is of interest therefore, that although the MS peripheral blood lymphocytes show little and varied response to stimulation with BP, it has been reported that stimulation with a soluble antigen from MS brain gives clearer differences in a rosette test for active T cells (36).

Summary

With the development of more refined techniques distinct progress has been made in the identification of likely participants in the immunological response in MS. Although the specificity of antibody in CSF and in the CNS is not known, anti-oligodendroglial antibody is present in the circulation. In addition, increased T cell activity is seen in the CSF which, as in EAE, may be related to a delayed hypersensitivity reaction. Unlike EAE, the sensitization may not be solely mediated by myelin BP. Other components in the inflammatory reaction are identified during a relapse. Thus there are small but definite changes in total PMNL neutral proteinase activity and evidence of release of lysozyme into the CSF of MS patients.

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Chapter 9

Suppression of Experimental Allergic Encephalomyelitis

Since experimental allergic encephalomyelitis (EAE) is a cell-mediated autoimmune disease it has been used as a useful model for the discovery and evaluation of immunosuppressive drugs. There are certain similarities between EAE and multiple sclerosis (MS) so that, as Levine and Sowinski (1) suggest, the response of EAE animals to drugs may serve as a guide to clinical drug therapy, perhaps an inadequate guide but better than none at all.

Treatment of EAE

Immunosuppression (Table 9.1) has been tried as a means of interfering with the development or expression of the immune response in EAE (2). Cytotoxic drugs such as methotrexate, a folic acid antagonist, or cyclophosphamide, an alkylating agent, suppress EAE in guinea pigs (3). These cytotoxic drugs act primarily at the proliferative phase after lymphocytes have contacted antigen but they can also moderate the blast reaction in target tissue as this also involves a burst of metabolic activity with active protein synthesis. The effect of ACTH and the corticosteroids on EAE has also been the subject of considerable study. Moyer and co-workers (4) found that 5 mg ACTH given daily from the day of inoculation to the 14th day suppressed clinical signs of the disease in guinea pigs. This has been confirmed in other species. When administration of these agents is delayed until a week or more after sensitization with adjuvant and brain antigen or until the onset of neurological signs, no suppression of the disease is noted (5). Kabat and co-workers (6) therefore suggested that these various agents only inhibit the inflammatory reaction to the adjuvant at the local injection site and draining lymph nodes, a reaction which is essential for production of the disease. It is further suggested that ACTH or corticosteroids have no effect on the basic immune process within the reticuloendothelial system, or on the disease process once it has been initiated within the central nervous system (CNS). However EAE in rabbits can be suppressed by appropriate large doses of methylprednisolone when treatment is delayed to within a few days or less of the time of onset of the disease in control animals (5). There is even some experimental evidence of clinical improvement when animals are treated after the onset of EAE. The suppressive effect does not continue long after the drug is discontinued, probably due to the persistence of antigen. These studies suggest that ACTH and corticosteroids have an effect in EAE both on the basic mechanism within the the reticuloendothelial system and to some extent on the disease process in the CNS.

Desensitization with Myelin Basic Protein

The best protection from EAE is achieved when basic protein (BP) is injected in Freund's incomplete adjuvant into animals before or at the time of inoculation with antigen in complete adjuvant (7,8,9). There is a parallel reduction in delayed-type cutaneous sensitivity to BP in these animals. This observation together with the importance of the dose of BP injected suggests that the underlying mechanism is desensitization of circulating effector lymphoid cells. In

TABLE 9.1 Possible Mechanism for Immunosuppression of EAE

1. Damage or elimination of resting T cells:
   Thymectomy, anti-lymphocytic serum, adrenocorticosteroids: (cortisone, methylprednisolone)

2. Desensitization of lymphocytes:
   Myelin basic proteins, peptides, copolymers

3. Inhibition of T cell metabolism or activation by antigen, by blocking nucleic acid or protein synthesis:
   Imuran, cyclophosphamide, methotrexate, X-rays, Levamisole, cyclosporin A

4. Prevention of contact of sensitized cells with antigen in target tissue
   Protective non-complement fixing antibody, desensitization of cells with passive antigen or non-specific mitogen; EN3638?

5. Reduction of lymphokine secretion:
   increased prostaglandins
   prostaglandin E
   linoleic acid
   Niridazole

6. Prostaglandin synthetase inhibition:
   Flumizole ineffective; Indomethacin increases inflammatory lesions

7. Interference with macrophage lysosomes -

   (a) stabilization of lysosomal membranes:
   glucocorticoids, chloroquine, phenylbutazone, aspirin

   (b) inhibition of active release of lysosomal enzymes:
   colchicine, vinblastine, cAMP

   (c) prior depletion of lysosomal content:
   Vitamin A and C deficiency

   (d) direct inhibition of lysosomal enzymes:
   gold salts, pepstatin (30)

After Arnason (2)

some cases remission of the disease has been effected when BP in Freund's incomplete adjuvant is given after initiation of the disease symptoms. Other non-encephalitogenic, basic, neural proteins have a similar desensitization effect (8) as have modified non-encephalitogenic myelin protein or peptide fragments (10). A peptide fragment, lacking tryptophan while producing circulating antibodies and delayed-type skin hypersensitivity to the BP, protects guinea pigs against EAE. Arnon and co-workers (11) have used copolymers of amino acids (e.g. alanine, glutamic acid, lysine and tyrosine) and obtained protection against EAE in guinea pigs and monkeys. The possibility that BP may serve to desensitize patients with MS was examined some years ago with no convincing success (12). However no adverse reactions were observed with the very low dose used and there are new initiatives to study the effect of large doses (30-75 mg per day by intradermal injection) in several centres.

The role of polyunsaturated fatty acids. It has been suggested that deficiency in brain polyunsaturated fatty acids (PUFA) may alter membrane composition and increase susceptibility to disease. Clausen and Møller (13) showed that diet deficient in linoleic acid and other essential fatty acids potentiated the action of injected brain homogenates in producing EAE in rats. This was later confirmed by Selivonchick and Johnston (14) who also showed that linoleate had a protective effect when fed to fat-deficient rats. These animals were fat-deficient from birth and therefore had a much more severe deficiency in brain and serum PUFA than is found in MS tissues. Since PUFA and their products (e.g. prostaglandins) may modify the immunological response linoleic supplements (0.5 ml/day) were fed to guinea pigs from 7 to 21 days after sensitization with adjuvant and BP (15). During this period there was a significant increase in serum linoleic acid concentration but no change in brain fatty acid composition. The treatment with linoleic acid had a mild protecting effect immediately before and during the time when clinical signs normally appear (Fig. 9.1). Oleic acid used as a control also had a significant protective action. The increased oleic acid concentration may modify linoleate levels in the serum which would account for its biological activity. When linoleate supplements were given from 7 days before to 7 days after inoculation with BP (that is ceasing a week before the expected appearance of clinical signs), there was no significant protection. This suggested that linoleic acid effects are short-lived. Only the regimen which was effective in suppressing EAE brought about suppression in the *in vitro* immune response to BP. However Meade and co-workers (15) failed to correlate *in vitro* lymphocyte reactivity to BP in the treated animals with the degree of clinical signs. Linoleic acid has been shown to have a weak protective action on graft rejection (16,17), and to suppress lymphocyte function *in vitro* (18). One possibility is that increased amounts of the prostaglandins of the E series are formed from the linoleic acids. These prostaglandins have pronounced immunosuppressive activity.

Anti-inflammatory Drugs

Various non-steroidal anti-inflammatory drugs have been tested for their ability to protect against brain damage in EAE. Salicylates and aspirin have been found to have some protective action but several other prostaglandin synthetase inhibitors have been found to be ineffective. For example, flumizole (4,5-bis(*p*-methoxyphenyl)-2-(trifluoromethyl)-imidazole) is a non-acidic trisubstituted imidazole which penetrates into the CNS; it is five times more potent than indomethacin *in vivo* in the carageenan-induced rat oedema test and is a powerful prostaglandin synthetase inhibitor. Nevertheless this compound is inactive when given for 11 days after the encephalitogen or may even increase the severity of clinical signs (Table 9.2) (19). This suggests that the drug is not effective

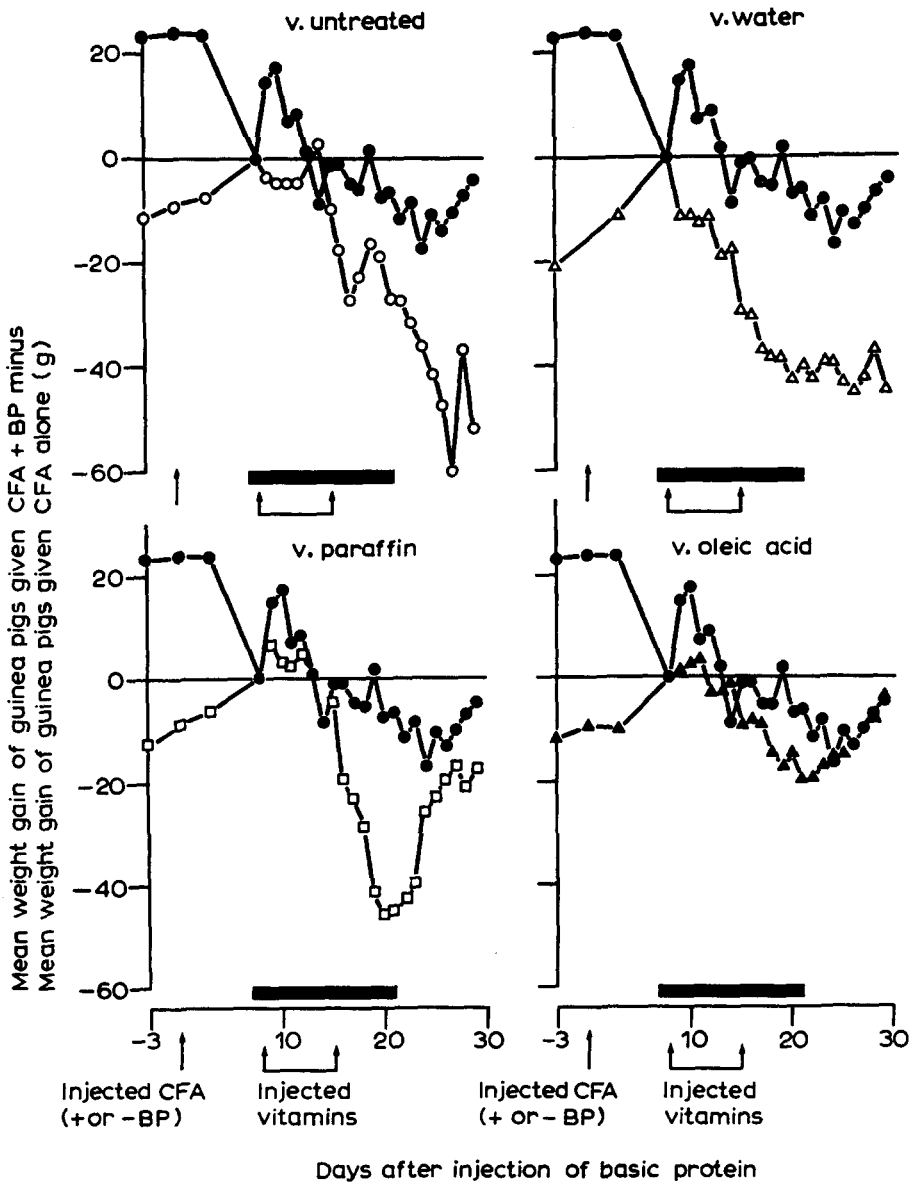

Fig. 9.1 Weight loss following injection of 10 μg BP in FCA: effect of various supplementary feeding schedules. The loss by linoleic acid fed animals (●) is compared with that by untreated animals (○); water fed animals (△); paraffin fed animals (□); and oleic acid fed animals (▲). The bars indicate the period of treatment. [Meade and co-workers (15)]. Reproduced with permission of J. Neurol. Sci.

during the events subsequent to interaction between the effector cells (lymphocytes) and neural antigen (BP).

TABLE 9.2 Effect of Flumizole on EAE

11 days after immunization	Average severity of clinical signs (days after start of therapy)								Deaths	Histology
	0	1	2	3	4	5	6	8		
1. Saline (by mouth x 5)	1.5	2.7	3.0	2.9	2.2	0.7	0.7	0.4	0/10	4
2. Flumizole (10 mg/kg by mouth x 5)	1.4	2.6	2.7	2.6	2.0	2.8	2.0	1.5	1/5	4
3. Flumizole (20 mg/kg i.p. x 5)	1.4	1.6	1.9	2.3	2.3	1.8	1.3	1.0	1/5	4
4. BP (1.4 mg/kg i.v. x 3)	1.4	1.5	1.0	0.4	0.2	0	0	0	0/5	3

After McIlhenny and co-workers (19)

Since essential fatty acids are prostaglandin precursors Mertin and Stackpoole (20) have examined the effects of combining supplementary feeding of EAE animals with linoleic and γ-linolenic acids with indomethacin. In all their experiments the fatty acid supplements (5-800 mg/kg daily) significantly suppressed the onset and severity of EAE. Indomethacin, presumably acting as an inhibitor of prostaglandin biosynthesis, abrogated the therapeutic effects of the unsaturated fatty acids. In our experiments (21) in guinea pigs with standard doses of spinal cord and adjuvant, anti-inflammatory drugs have been found to intensify the histological damage and clinical signs of the disease in relation to their activity as prostaglandin synthetase inhibitors. Thus in the acute experimental animal it appears that prostaglandins may be exerting a protective effect, perhaps by the mechanism suggested by Morley (22).

Drugs Affecting the Cellular Response

Levine and Sowinski (23) have examined the action of EN3638 [6-hydroxyphthalaldehydic acid, O-(p-chlorobenzyl) oxime], a new oxime derivative of salicylic acid, in suppressing EAE. Lewis rats with acute or hyperacute EAE respond to the protective effect of the drug (Table 9.3). When high doses (250 mg/kg) are given orally three times a week, from the time of inoculation, there is complete protection from neurological signs; with smaller doses (150 mg/kg) signs develop after 16 days rather than after 7 days as in the untreated group. Experiments on adrenalectomized rats exclude the possibility that the suppressive effect is adrenal-mediated. In single-dose experiments with rats it appears that the drug

TABLE 9.3

Suppression of hyperacute EAE in Lewis rats by intermittent drug treatment for 25 days. EN3638: [6-hydroxyphthal-aldehydic acid, O-(p-chlorobenzyl) oxime].

Dose mg/kg	Times per week	Day of onset	Mortaolity
Vehicle	5	7.1 ± 0.3	9/10
150	3	16.2 ± 0.7	0/5
150	5	24.7 ± 0.5	0/3
250	2	12.8 ± 3.2	4/9
250	3	none	0/9
400	2	none	0/9

The antigen was guinea pig spinal cord. [Levine and Sowinski (1)].

had its greatest effect when given 6 days after the encephalitogen. The onset of lesions and mortality were not affected when the drug was given on the 7th or 8th day. Thus EN3638 has its greatest impact at a stage immediately before histological signs of damage first occur (i.e. day 7). On the other hand, salicylates (300 mg salicylate/kg s.c. or 400 mg/kg of calcium carbaspirin by mouth) have no protective action when given 6, 7 or 8 days after immunization, although these substances give some protection when treatment begins earlier. Passive transfer experiments suggest that the drug prevents and even reverses sensitization to neural antigens in the donor rat 6 to 8 days after inoculation. EN3638 had only slight effect on fully sensitized lymphoid cells, or on the recruitment of non-immune inflammatory cells in the nervous system. When recipient rats were treated with EN3638, there was relatively little protection (lesion score 1.7 compared to 2.5 in untreated animals). In addition, pretreatment of recipient Lewis rats with cyclophosphosphamide inhibits transfer of EAE with sensitized donor lymph node cells (1). The anti-schistosomiasis drug, Niridazole, has recently been found to inhibit T cell activity selectively. Sera from guinea pigs given the drug (i.e. containing metabolites), blocked reversibly the production of antigen-induced migration inhibitory factor (MIF) by sensitized guinea pig lymph nodes (24,25,26). Niridazole (100 mg/kg body weight) given orally reduces the clinical signs and histological damage produced by EAE in mice (Table 9.4) (26). The drug is most effective when treatment is begun before inoculation of antigen and protection lasts up to 10 weeks. However in the treated mice there was no effect of niridazole on either release of MIF in response to BP, or on production of antibody, or on the non-specific inflammatory reaction to adjuvant. The small peptide fungus metabolite cyclosporin A appears to act selectively (27,28) on T cells and has low cytotoxicity for haemopoietic tissues. Experiments on kidney and cardiac allografts suggest that the peptide may be able to eliminate lymphocyte clones which have responded to a specific antigenic challenge, leaving intact clones to respond to other challenges such as virus infections (28,29). The incidence of paralysis in susceptible rats, inoculated with spinal cord plus adjuvant, was significantly reduced when oral doses of 50 mg of cyclosporin A were given daily (27).

TABLE 9.4

Effect of Niridazole (100 mg/kg body weight) on signs of disease and damage in mice with EAE

Treatment schedule	Number	Number of mice with					
		signs of disease			Onset of EAE mean time	lesions	
		mild	severe	death		mild	severe
3 times weekly, 2 weeks before and after immunization	20	2	2	0	16	4	2
3 times weekly, for 2 weeks after challenge	8	2	4	0	15	2	4
Control (saline)	25	0	25	5	12	4	16

After Bernard and co-workers (26)

Summary

Desensitization of EAE has been achieved by pretreatment with myelin BP. Immunosuppression with antimetabolites (e.g. cyclophosphamide) and other inhibitors of lymphocytic protein synthesis are found to be effective in increasing the time of onset and the severity of the clinical signs of the disease. It also seems that enhanced synthesis of prostaglandins may serve to protect animals as PUFA supplements modify and prostaglandin synthetase inhibitors exaggerate the paralytic and histological effects. This may explain the slight improvement seen in MS patients taking linoleic acid.

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Chapter 10

Conclusion

Epidemiological evidence and some morphological observations suggest that an infective agent, acquired during childhood, is responsible for multiple sclerosis (MS). A clue to the nature of such an 'infective agent' could come from specificity studies on antibody found in the cerebrospinal fluid (CSF) of patients or from IgG isolated from affected post-mortem brain, and it seems possible that the agent may be one or more viruses, possibly of the paramyxovirus group. So far only a small proportion of the oligoclonal bands has been found to be directed against measles or other viruses but other types of antigen may be responsible. Little antibody to myelin basic protein has been detected though there are circulating antibodies to oligodendroglial cells in the blood during a relapse of MS. There are reports of an astrocytic response in apparently unaffected parts of the white matter but the earliest biochemical change in presumptive lesions is increased lysosomal activity with loss of the myelin BP. BP is highly sensitive to proteolytic attack and when fragments of it are released into the cerebrospinal fluid (CSF) at the time of an exacerbation the antigenically active residues may serve to sensitize peripheral lymphocytes or alternatively to function as immunoregulators (1). Some of the BP sequence is homologous with a fibroblast growth factor (2) and thus BP fragments may also be responsible for activating astrocytes in the region of the plaque. During a relapse the serum of MS patients contains an unidentified complement-dependent antibody which has been shown in vitro to have a neuroelectric blocking activity. This factor may be responsible for some of the reversible signs seen during the course of the disease. Many MS sufferers possess a characteristic tissue antigen type which is possibly associated with a difference in the nature of the immune response of the individual compared to unaffected controls. Thus in MS patients, there are apparent defects in the delayed hypersensitivity reaction. Indeed, changes in the kappa:lambda ratio of CSF antibodies may also indicate some defect in immunological regulatory mechanisms.

Increased neutral proteinase activity of polymorphonuclear leucocytes in the blood during an attack of MS probably relates to the inflammatory reaction frequently found around blood vessels in the demyelinated central nervous system. There is the concomitant appearance in the CSF of cell bound neutral proteinase activity and release of lysozyme. The cellular immune response seems to be particularly important for sensitized T cells are found in raised concentration in the CSF of MS patients close to a relapse. These findings on the delayed hypersensitivity reaction have drawn attention to the relevance of experimental allergic encephalomyelitis (EAE) as a model of the inflammatory reaction component in MS. The finding of a relapsing remitting type of chronic EAE adds to the significance of this as an experimental tool. Nevertheless, there are important differences - for example, the non-involvement of the myelin BP in the induction of the initial lesion in MS and the relative lack of demyelination in the acute EAE (but not in the chronic condition). There is also intriguing evidence of clinical signs in acute EAE being dissociated from the presence or absence of neuropathological lesions (Table 10.1).

TABLE 10.1 Immunological Responses in EAE and MS

	EAE	MS
Humoral		
Elevated CSF IgG and pleocytosis	+	+
BP fragments in CSF	+	+
Anti-BP antibody		
(a) serum	+	-
(b) CSF	+	±
IgG in lesions	+	+
Serum demyelinating and anti-neuronal activity	+	+
Cell-mediated		
MIF for macrophages (stimulation by myelin BP)	+	∓
Lymphoblast formation " " " "	+	∓
% Active T-cells decreased in blood and increased in CSF	+	+
Neuropathology		
Perivascular cuffing	+	±
Demyelination	∓	+

Although the inflammatory reaction is only one factor in the pathogenesis of MS and perivascular cuffing is not invariably associated with demyelinating lesions it may be possible to alleviate a relapse by using drugs which have proved effective in suppressing EAE. Apart from those immunosuppressants already tested in clinical trials there are now new anti-inflammatory drugs which act at different points in the inflammatory reaction. Some substances (e.g. prostaglandin synthetase inhibitors) may actually increase disease activity but others, for example, those selectively acting on T-lymphocytes or substances stimulating prostaglandin synthesis, may prove of value. Unfortunately it is unlikely that any such treatment would be effective in chronic MS cases where degenerative change already predominates.

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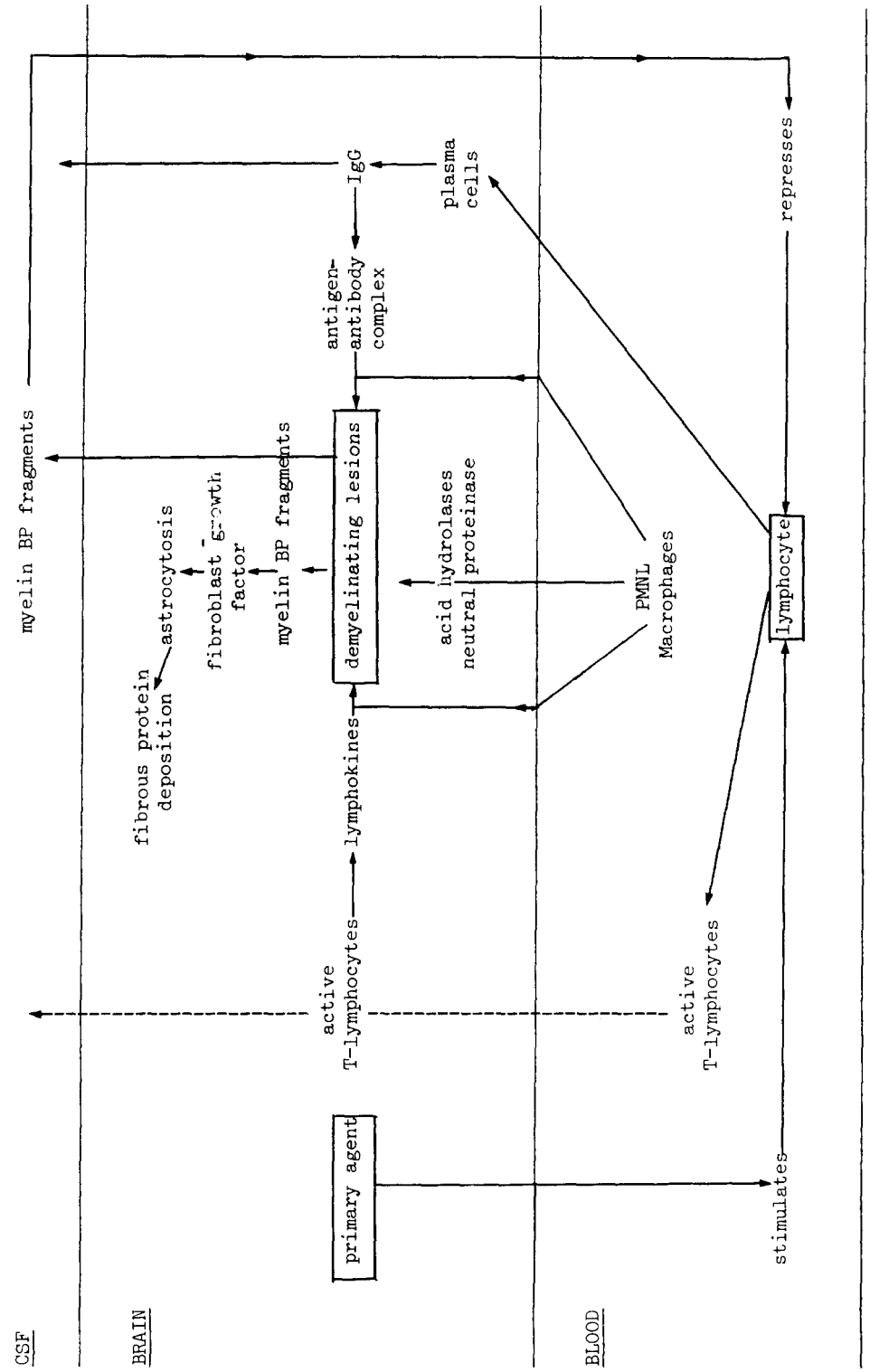

Fig. 10.1 Postulated changes in CSF, Brain and Blood in MS

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